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Consequences of within-family communication and rearing environment on offspring performances and parental investment in two bird species

Ph.D. Thesis

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GENERAL INTRODUCTION

Introduction

Understanding the causes and mechanisms underpinning individual phenotypic variation, which is the ultimate determinant of differential contribution of individuals to the genetic composition of the subsequent generations, is crucial for any research field in evolutionary ecology (Williams, 1992). While environmental conditions can have obvious, immediate consequences for phenotype, in recent years increasing attention has been devoted to the long-term effects of the conditions experienced during early phases of life, typically spanning from conception to developmental maturity, on survival and reproduction (see Lindström, 1999; Metcalfe and Monaghan, 2001; Ricklefs et al., 2006). Perinatal environment is mainly determined by parental decisions over the time and place of breeding, which can affect e.g. food abundance and/or the presence of parasites and predators, as well as over the number of offspring produced and the effort devoted in attending the progeny, which can determine the intensity of within-brood competition and ultimately the amount of resources received by each member of the progeny.

The aim of the present dissertation is to examine diverse factors that can contribute to influence variation in the quality and viability of the offspring before the independence from parents, and potentially having an important role in affecting individual performance into adulthood, using two altricial bird species as model organisms. More specifically, this work is concerned with the effects of the within-brood competitive environment and the unequal parental investment towards different members of the progeny on offspring growth and phenotypic quality, with special focus on intra-familiar conflicts mediated by the so-called begging behaviour. It further investigates the potential consequences, in terms of reproductive success, of the conditions experienced during development, typically mediated by parental breeding decisions.

1.1 Parent-offspring conflict over parental investment

In species where the offspring are totally dependent on the resources, particularly food, provided by parents, evolutionary conflicts of interest within the family are expected to arise because individual family members disagree over the allocation of parental investment (Clutton-Brock, 1991; Mock and Parker, 1997). Since the pioneering works of Hamilton (1964) and Trivers (1974), theoretical models have emphasized that intra-familiar conflicts occur as a consequence of sexual reproduction, because family members share only part of their genes (e.g. Macnair and Parker, 1979; Parker and Macnair, 1978; 1979; Parker et al., 1989; 2002; Godfray, 1995a). In diploid animals adopting sexual reproduction, parents are not genetically identical to their offspring: the coefficient of relatedness (i.e. the proportion of the gene pool shared between two individuals) of each parties with itself is 1.0 compared to 0.5 with its counterpart (Hamilton, 1964). Although the different parties in a family share common evolutionary interests, benefits and costs of parental investment are divergent between parents and offspring (Trivers, 1974; Clutton-Brock, 1991; Mock and Parker, 1997). Given that a fixed amount of

parental investment results in different fitness payoffs for offspring and parents, and that natural selection is therefore expected to act in conflicting ways on genes expressed in parents and on those identical by descent expressed in offspring, a parent-offspring conflict over the amount of parental care that each individual member of the progeny should receive is expected to occur (Trivers, 1974; Clutton-Brock, 1991). Furthermore, models of parental care (see Clutton-Brock, 1991 for an exhaustive overview) argue that parental additional investment in a given brood increases its reproductive value, but also results in a decrease of the future reproductive success of the parents. In general, it is widely acknowledged that parents are selected to invest their limited resources in relation to their own interest, by balancing expenditure in the current reproduction with other present and future activities (Trivers, 1974; Clutton-Brock, 1991; Godfray, 1995a), and maximizing the difference between benefits and costs of parental care towards any single offspring (Trivers, 1974; Clutton-Brock, 1991). On the other hand, offspring are selected to obtain from their parents more care that it would be optimal for them to provide (Trivers, 1974; Macnair and Parker, 1979). Opposing selective pressures on either generation should thus favour the evolution of different strategies to prevail in such an 'intra-familiar battleground', with offspring trying to manipulate both the amount (i.e. the overall investment in a given brood) and the allocation (i.e. the investment in each member of the current brood) of parental investment to their advantage and parents limiting extra-expenditure in care which is solicited by the offspring in order to maximize their own fitness. Theoretical models focused on the resolution of parent-offspring conflict (e.g. Trivers, 1974; Macnair and Parker, 1979; Parker and Macnair, 1978; 1979; Johnstone, 1999; 2004) have demonstrated that evolutionary stable strategies can arise at intermediate levels of parental investment between those expected to maximize parental or, respectively, offspring fitness (but see Alexander, 1974; Godfray, 1991; 1995b; see also **Paragraph 1.4**). Most of these models also envisage parent-offspring conflict as mediated by the so-called begging behaviour, consisting of a diverse set of morphological and behavioural displays that offspring use to solicit provisioning of care (e.g. Godfray 1995a; Kilner and Johnstone, 1997; Johnstone, 1999; 2004; see also **Paragraph 1.4**).

Given that parents are equally related to all their biological descendants, they should theoretically promote an even share of resources among offspring, unless asymmetry in reproductive value among siblings are otherwise generated by e.g. parasitism or birth order (Hamilton, 1964; Trivers, 1974; Smith and Fretwell, 1974; Clutton-Brock, 1991; but see Temme, 1984; Haig, 1990; Lessells, 1998; 2002; see also **Paragraph 1.3**). Conversely, each offspring shares twice as much of its gene pool with itself than with any other full sibling, and is therefore selected to increase its share of parental resources to the expense of its siblings, leading to competition among siblings for the access to resources provided by parents (see **Paragraphs 1.2** and **1.4**). Such a disagreement over the allocation of parental resources leads to two main forms of parent-offspring conflict, that are expected to occur in relation to the species-specific life-histories: inter-brood conflict arises whenever parents alter the parental investment in the current brood as a

response to the offspring demands, thus affecting their available resources to be allocated to future breeding attempts (Trivers, 1974; Parker et al., 1989; Clutton-Brock, 1991; Mock and Parker, 1997). Hence, this type of parent-offspring conflict occurs in multivoltine species and involves offspring of different broods. In addition, when brood size is greater than one, different requests by individual siblings may cause a deviation from the optimal investment for parents in each offspring within brood (Macnair and Parker, 1978; Parker et al., 1989; Clutton-Brock, 1991; Mock and Parker, 1997). This conflict among brood mates, the so-called intra-brood conflict, is typical of multiparous species and can occur even without any difference in the total amount of parental investment towards the whole brood. Parent-offspring intra-brood conflict can be thus measured as the difference between the investment per offspring that is optimal for the parents and the skews introduced by the interactions among siblings (see **Paragraphs 1.2** and **1.4**).

1.2 Sibling interactions over the allocation of parental care

In a ideal world, parents would experience no uncertainty about the optimal size of their family: under conditions of no zygote failure, no offspring mortality and perfectly predictable resources abundance, they would simply produce the exact number of offspring they would be able to successfully rear under the current environmental conditions. Nevertheless, parents in the real world are faced with various major problems, spanning from the enormous variation of resources availability to offspring mortality unrelated to parental performance, that can severely affect the ideal offspring production. A general strategy adopted by parents of disparate, non-phylogenetically related, taxa to cope with unpredictable variation in environmental conditions and potential offspring mortality is an initial over-production of progeny, followed by a secondary adjustment of the brood size via mortality of part of the brood, the so-called brood reduction (Lack, 1954; O'Connor, 1978; Haig, 1990; Mock, 1994; Mock and Forbes, 1995; Mock and Parker, 1998; see also **Paragraph 1.3**). The amount of both over-production and 'sacrifice' of the progeny, and the way whereby parents adjust family size widely vary across species, but also across population within a species, according to life-histories and environmental conditions to which they are exposed (O'Connor, 1978; Forbes, 1993; Mock, 1994; Mock and Forbes, 1995; Mock and Parker, 1997; 1998).

Although such general strategy seems costly for parents because it entails a waste of parental investment, since any dead offspring constitute a loss in fitness, theoretical studies have proposed at least three, not mutually exclusive, evolutionary hypotheses for the adaptive significance of progeny over-production (Mock and Forbes, 1995; Mock and Parker, 1997). All proposed mechanisms identify 'core brood', representing the number of young that can normally be raised to independence, and 'marginal brood' members, which can be physically indistinguishable or not from the core ones but are anyway less valuable in terms of fitness return to parents (Mock and Forbes, 1995; Forbes, 2007; see also **Paragraph 1.3**). The Resource-tracking Hypothesis (Temme and Charnov, 1987) posits

that parents produce an 'optimistic' brood size in order to maximize reproductive success under unpredictably favourable environmental condition during breeding (see also Mock 1994; Mock and Parker, 1998). According to the Offspring Facilitation Hypothesis, marginal offspring can contribute to the fitness of siblings by helping the core part of the progeny to survive and/or reproduce. This evolutionary mechanisms has been initially envisaged in the context of sibling cannibalism (see Elgar and Crespi, 1992), whereas, at the opposite, it has been recently suggested that the presence of a 'thrifty' offspring can favour brood mates indirectly (e.g. maintaining an high parental food provisioning rate or an high temperature in the nursery), leading to the evolution of a sort of sibling mutualism (Forbes, 2007). Finally, the Replacement Offspring Hypothesis posits that marginal offspring can function as substitutes of the more valuable siblings whenever the survival of the latter is impaired by external factors, like predation or disease (Dorward, 1962; Forbes 1990; 1991; 1993).

Although the total parental expenditure in a given brood usually increases with its size, the *per capita* share of care obtained by individual offspring is expected to decline with increasing number of heads to be fed (see Clutton-Brock, 1991; Mock and Parker, 1997 for exhaustive overviews), ultimately affecting the offspring growth rate (see Ricklefs, 1982; Rodríguez-Gironés et al., 2001). The amount of resources received by each member of the progeny, combined with the conditions experienced during the rearing period, is crucial in determining its growth, physiology and viability (Ricklefs, 1982), with potential consequences that can eventually carry-over into adult life (see e.g. Searcy et al., 2004; Blount et al., 2006; Naguib and Nemitz, 2007). Competition among siblings occurs when brood mates are constrained to share a limited space and pool of resources, therefore it is mainly concentrated from birth to dispersal (Mock and Parker, 1997; 1998). From the point of view of individual offspring, each sibling can thus be seen as a dangerous competitor for the limited resources provided by parents and therefore represents a threat for its survival prospects. From this starting point common to all multiparous species, however, a wide range of sibling rivalries can arise according to the life-history of different species and rearing conditions, ultimately resulting in fairly different outcomes of sibling interactions, spanning from sibling cannibalism to scramble competition for resources (see Mock and Parker, 1997 for an exhaustive overview). In birds and mammals, competition among siblings for parental resources is the norm, and can often be lethal to part of the progeny, either because of physical aggression leading to siblicide (see Mock et al., 1990; Godfray and Harper, 1990; Mock and Parker, 1998; Drummond, 2002), or because of starvation of young and/or weak offspring, whose access to parental resources is restrained by stronger siblings (Howe, 1976; Sullivan, 1989; Cotton et al., 1999). This may occur, for example, by jostling for the position that assures the largest share of food and/or scrambling mediated by begging behaviour (sensu Kilner and Johnstone, 1997; Mock and Parker, 1997; see also **Paragraph 1.4**).

On the other hand, however, siblings can also be considered as precious carriers of a part of own genome, and thus as important contributors in increasing the indirect component of own inclusive fitness (Hamilton,

1964). The Inclusive Fitness Theory (Hamilton, 1964) sets an evolutionary upper limit to selfishness among siblings: an 'altruistic' act should in fact be performed when the indirect fitness gain obtained because of such an act exceeds the direct fitness cost paid in performing it (Hamilton, 1964). In mathematical terms, a reduction of selfishness is expected to occur whenever $rB \geq C$ (the Hamilton Rule), where B is the net additional fitness benefit gained by the recipient of the 'altruistic' act, C is the fitness cost to the performer of the act, and r is the coefficient of relatedness between the two individuals, coinciding with the proportion of their genes identical by descent (in the case of full-sibling r is 0.5). Therefore, C and rB represent, respectively, the direct and indirect fitness consequences of an act. The Hamilton Rule has been used to explain a wide variety of social interactions and the existence of several behavioural traits that impose a direct fitness cost to the actor, ranging from alarm calls to cooperative breeding and courtship strategies (see Foster et al., 2006; Gardner, 2011 for reviews; but see West et al., 2002). In the context of sibling interactions, an offspring is therefore expected to reduce consumption of common resources when the next unit of parental investment would become more than twice as beneficial to its full-sibling's personal fitness than to its own.

As the marginal benefit of acquiring an additional unit of parental investment is predicted to decrease with increasing individual condition (Godfray, 1991; 1995b; Forbes, 2007), offspring have to trade the indirect fitness cost of subtracting resources to their siblings against the direct gain of obtaining more parental resources. Under an inclusive fitness perspective, condition-dependent strategies by different members of the progeny are expected the evolve: offspring should thus be selected to shift their behaviour from vigorous competition, potentially leading to the death of sibling(s), to moderation of selfishness in relation to direct and indirect fitness payoffs. The strategy adopted mainly depends on the ecological (e.g. abundance of resources) and social (e.g. number of competitors) conditions to which offspring are subjected during the rearing period, as well as on the offspring rank the brood hierarchy (O'Connor, 1978; Parker et al., 1989; Mock and Parker, 1998; see also **Paragraph 1.4**). In extreme cases, a sibling can be more valuable as a food item rather than as a carrier of own genes: when resources are scant and sibling's reproductive value is low anyway, the siblicide followed by the consumption of a potentially viable brood mate can be favoured (O'Connor, 1978; Elgar and Crespi, 1992). Conversely, offspring in good condition, that can benefit from future reproduction of their kin, should be prone to assure a large share of food to their siblings in poor state. However, few empirical studies have provided evidence of altruistic behaviour among siblings over the allocation of parental resources: for example, Stamps et al. (1985) have documented that large and well-fed nestling budgerigars (*Melopsittacus undulatus*) may actively feed their smaller needy siblings.

1.3 Differential parental investment and the Sex Allocation Theory

The prediction that parents should invest an even amount of resources in each offspring because they share with them an equal

proportion of genes is tenable only if all members of the progeny have the same reproductive value (Smith and Fretwell, 1974). Whenever this condition is not fulfilled, parents can maximize their fitness by preferentially allocating their investment towards a part of the progeny (Temme, 1986; Haig, 1990). Offspring can differ in quality and/or in survival prospects (see also the concepts of 'core' and 'marginal' offspring in **Paragraph 1.2**), and can thus differentially contribute to parental fitness: parents are thus expected to bias their investment according to individual offspring quality so that the marginal benefits from additional investment would be equal for each offspring (Temme, 1986). Parents can intervene in adjusting investment towards individual offspring at different times, from the conception to the dispersal of the progeny (Clutton-Brock, 1991; Mock and Parker, 1997; Glassey and Forbes, 2002a). They can firstly generate asymmetries in offspring phenotype at birth, by allocating differential amount of resources to different embryos (Moussesau and Fox, 1998) or, in birds, by promoting hatching asynchrony (see Magrath, 1990; Stenning, 1996) that determines a hierarchy among competing offspring (see the concepts of 'core' and 'marginal' offspring described in the **Paragraph 1.2**). This initial inequity can later be used as a basis for adjustment of parental investment, by for example providing more food to the more valuable offspring (see Mock and Forbes, 1995; Stenning, 1996; Mock and Parker 1997; 1998; Glassey and Forbes, 2002a). Evolutionary theory of parental investment therefore posits that natural selection should have favoured the evolution of the ability of parents to assess offspring quality, by using phenotypic traits (e.g. relative body mass) that can reflect reproductive value (Kilner, 1997; Saino et al., 2000a; 2000b; 2003a; de Ayala et al., 2007; Dugas, 2009), or by exploiting reliable signals (e.g. visual and acoustic begging displays) produced by offspring to advertise their need and condition (Grafen, 1990; Godfray, 1991; 1995b; Johnstone, 1999; review in Kilner and Johnstone, 1997; see also **Paragraph 1.4**).

One important source of variation in offspring reproductive value is sex, given that sons and daughters may differ in embryo development (Sellers and Perkins-Cole, 1987; Cook and Monhagan, 2004), in susceptibility to rearing conditions or parasites (Nager et al., 2000; Tschirren et al., 2003; Bize et al., 2005; Chin et al., 2005), and have different demand of resources because of sexual dimorphism or marked differences in physiology, both resulting in a sex-specific daily energy expenditure or in a differential assimilation of food (Martins, 2004; Vedder et al., 2005; see Uller, 2006 for a review). In addition, male and female offspring may differently benefit from current environmental and/or demographic circumstances (Greenwood, 1980; Martín et al., 2008; Dreiss et al., 2010a). In sexual dimorphic species, for example, the bigger sex may show a greater susceptibility to food shortages (e.g. Teather and Weatherhead 1989; Arroyo et al., 2002), whereas it can be advantaged in size-mediated competition for food (e.g. Fargallo et al., 2003).

Despite frequency-dependent selection should promote a balanced offspring sex ratio (i.e. the proportion of male to female individuals) (Fisher, 1930), the Sex Allocation Theory predicts that can be favoured in producing more offspring of the sex that

provides the highest fitness gain (Trivers and Willard, 1973; Charnov, 1982; Frank, 1990; see Hardy, 2002 for an extensive overview). Adaptive sex ratio adjustment are well documented in haplodiploid species (Godfray, 1994; Hardy, 2002), while the application of Sex Allocation Theory to vertebrates with chromosomal sex determination is still greatly debated and controversial (Cockburn et al., 2002; West and Sheldon, 2002; Ewen et al., 2004; Cassey et al., 2006). Nevertheless, evidence of non-random sex allocation consistent with expectations from theoretical models has been provided in mammals (e.g. Clutton-Brock et al., 1984; Kruuk et al., 1999; see also a review in Cameron, 2004) and birds (e.g. Komdeur et al., 1997; Kilner, 1998; Nager et al., 1999; Saino et al., 2010; see also reviews in Hasselquist and Kempenaers, 2002; Komdeur and Pen, 2002).

Although various constraints should limit the possibility of skewing offspring primary sex ratio, natural selection is expected to favour the evolution of mechanisms whereby parents can preferentially produce progeny of either sex (see Uller, 2006). Indeed, several experimental studies have suggested potential mechanisms used by parents (mostly by the mother) to adjust both primary (i.e. at the time of conception) and secondary (i.e. between birth and dispersal) sex ratio (reviewed in Krackow, 1995; James, 1996; Oddie, 1998; Hasselquist and Kempenaers, 2002). Such possible mechanisms have been studied particularly in birds, where females, which are the heterogametic sex, could manipulate primary sex ratio by preferentially producing gametes containing either of the sex chromosomes (Correa et. al., 1995; Badyaev et al., 2005), by allocating different amount and quality of resources to male and female eggs (Pike and Petrie, 2005; Badyaev et al., 2006; Saino et al., 2010), or by adjusting the laying sequence of the eggs containing male or female embryos, thus generating a competitive dominance among nestlings of different sex that can be subsequently used to bias the secondary sex ratio (Badyaev et al., 2006). Furthermore, mothers (or parents in species with biparental nestling care) could provide different food supplies to sons and daughters (Lessells, 1998), or tolerate asymmetric competition mediated by e.g. within-brood size hierarchy and competitive ability (Arroyo et al., 2002; Fargallo et al., 2003), ultimate resulting in sex-biased mortality before dispersal.

1.4 Begging behaviour and the resolution of intra-familiar conflicts

Begging typically includes a number of acoustic, behavioural, morphological and chemical displays addressed by altricial young to their parents in order to solicit the provision of food or other critical resources (see Clutton-Brock, 1991; Kilner and Johnstone, 1997; Mock and Parker, 1997; Wright and Leonard, 2002). The evolutionary function of begging in mediating the resolution of parent-offspring conflict and sibling competition over care allocation has been widely debated in the last decades, and many theoretical models have been proposed (e.g. Macnair and Parker, 1979; Parker and Macnair, 1978; 1979; Godfray, 1991; 1995b; Rodríguez-Gironés et al., 1996; Johnstone, 1999; 2004; Royle et al., 2002; Johnstone and Roulin, 2003). In particular, in the context of the evolutionary games between

offspring and parents, begging behaviour is considered crucial in affecting the two key parental decisions about the amount of resources invested in current compared to future broods (provisioning, affecting inter-brood conflict) and in each individual member of the current progeny (allocation, affecting the intra-brood conflict).

All models focusing on the resolution of intra-familiar conflicts are underpinned by three main assumptions (see Mock and Parker, 1997; Kilner and Johnstone, 1997; Johnstone and Godfray, 2002; Royle et al., 2002). First, begging is influenced by additional resources received by offspring: in particular, begging intensity decreases when offspring get fed. Second, parents supply resources according to begging intensity: higher begging intensity therefore secures the offspring with a larger amount of resources, which in turn increases its direct fitness component. Finally, begging displays impose a cost to offspring: such cost increases as begging intensity increases, thus preventing disproportionately large offspring solicitation.

Historically, two different theoretical approaches to how begging behaviour can affect parental care can be identified, which mainly differ in the role that parents and offspring are assumed to play in the proximate control of allocation of care (see Kilner and Johnstone, 1997; Johnstone and Godfray, 2002; Royle et al., 2002; Mock et al., 2011). According to the first perspective, parents can passively accept the outcome of begging intensity escalation among competing offspring, making the only 'passive choices' of allocating resources to the individual member of the progeny which has displayed the most conspicuous stimulus (but it applies also to situations with a single offspring). In the so-called Scramble Model, begging behaviour has evolved as a mechanism to manipulate parental food provisioning, whereby offspring try to obtain from parents more resources than it would be optimal for parents to provide. Such a 'pro rata' model, developed by Macnair and Parker (1978; 1979), assumes that parents respond to higher levels of begging by providing more food, and that offspring beg less if fed more, with both such responses being fixed. In other words, parents respond to an increase of begging solicitation by enhancing food provisioning to prevent further expensive displays that can compromise offspring fitness. At evolutionary equilibrium, the 'pro rata' model predicts that the amount of resources obtained by offspring is a compromise between the optima for parents and offspring.

Conversely, in the Honest Signal of Need Model, parents have the power of 'active choice', because they can base decisions about the amount of resources to be invested in individual offspring largely on reliable information of offspring need conveyed by begging displays (Godfray, 1991; 1995b; see also Johnstone and Godfray, 2002). Godfray's model (1991; 1995b) stipulates that offspring can vary in their condition, both in the short-term (i.e. hunger level; but see Mock et al., 2011) and in the long-term (i.e. offspring quality, that can be measured as the total amount of resources needed by an individual to reach the independence from parents), which in turn cannot be directly determined by parents. In addition, the model assumes that the marginal benefit of receiving an additional fixed amount of food and the cost of performing begging solicitation at a fixed level

increase as the offspring condition decreases. Information conveyed by begging displays is thus reliable in signalling offspring need, because the high cost in performing them enforces honesty on advertisement of need. The costly nature of begging signals thus prevents any begging escalation by offspring in good condition, because they would not gain any positive marginal fitness return from obtaining additional resources. Hence, by exploiting begging displays parents can adjust food allocation according to the pattern that maximizes their own fitness. This argument can thus be seen as an application of the handicap's principle proposed by Zahavi (1975) in the context of the evolution of sexual selected traits, and applied by Grafen (1990) to the evolution of biological signals. In the Honest Signal of Need Model parents therefore always 'win' the parent-offspring conflict because each offspring receives an amount of parental investment coinciding with the parental optimum, whereas it necessitates a costly signalling system that depresses the fitness of the offspring and, consequently, the fitness of parents.

A slight different version of this model, also assuming a parental active choice and begging as reliable indicator of offspring quality, has been proposed verbally by Mock et al. (2011). According to such a Signal of Quality Model, explicitly based on the work of Grafen (1990), parents are expected to skew the investment towards offspring that advertise their large reproductive value (i.e. large fitness return for parents) by condition-dependent signals. The Signal of Quality Model, using the words of Mock et al. (2011), 'minimizes the parents' wasting on lost causes' and it can be considered a case of the Godfray's model that fits well to conditions of food shortage and to species with high occurrence of brood reduction (Mock et al., 2011; see also **Paragraph 1.3**).

Although being based on a different role of begging, which is considered as reliable signal of offspring state or not, the two approaches provide similar conclusions (both conceptually and mathematically) about the cost of begging and about the outcome of allocation decisions, with individual offspring that beg the most receiving the largest share of resources. Such conclusions are supported by a plethora of correlative and experimental studies on different taxa, from invertebrates to birds and mammals, demonstrating that begging is costly in terms of energy expenditure (e.g. Kilner, 2001; Rodríguez-Gironés et al., 2001; Moreno-Rueda and Redondo, 2011; Boncoraglio et al., 2012; but see Martín-Galvez et al., 2011; see also Wright and Leonard, 2002) and overall predation risk (e.g. Haskell, 1994; Leech and Leonard, 1997; McDonald et al., 2009; see also Wright and Leonard, 2002), and that parents provide resources according to the intensity of begging (reviewed in Kilner and Johnstone, 1997; Wright and Leonard, 2002; Kilner and Drummond, 2007; Mock et al., 2011). Furthermore, strong empirical evidence supports the assumption that begging can reliably convey information about offspring need both in the short- and in the long-term (see Wright and Leonard, 2002). The short-term need is mainly related to the level of satiation (e.g. Kilner, 1995; Leonard and Horn, 1998; 2001a; Saino et al., 2000b; Dor and Lotem, 2011; but see Mock et al., 2011), while differences in long-term need can arise from various sources, including body size (Kilner, 1995; Kacelnik et al., 1995; Cotton et al.,

1999), parasite burden (Christe et al., 1996; Saino et al., 2000b), hatching asynchrony (Zach et al., 1982; Müller et al., 2003), and maternal effects (von Engelhardt et al., 2006; Loiseau et al., 2008), which potentially reveal offspring reproductive value (see e.g. Saino et al., 2000a; 2003a; de Ayala et al., 2007; see also **Paragraphs 1.2**, **1.3** and **1.6**).

In the practice, however, testing whether begging has evolved as a reliable signal to parents or as a consequence of a scramble between parents and offspring is difficult. In uniparous species a signaling function of begging seems more likely. However, although the Godfray's argument can be applied also when offspring are produced in brood (see Godfray, 1995b), in such circumstances several form of selfishness can intervene in subverting parental authority (Parker et al., 1989; Mock and Parker, 1998), possibly resulting in a marked deviation from the allocation that would correspond to the parental optimum. It is likely that several real-world instances are best explained as a compromise between the two different models (see Mock and Parker, 1997; 1998; Johnstone and Godfray, 2002), with parents trying to maximize their own fitness and offspring managing to influence parental allocation decisions by, for example, keeping the position in the nest that assures the largest share of food (see e.g. Kacelnik et al., 1995; Kilner, 1995; Cotton et al., 1999).

Competition among siblings is therefore considered as a major determinant of begging behaviour and of the outcome of food allocation: begging levels are in fact expected to increase with the number of competitors (Macnair and Parker, 1979; Godfray, 1995b; Rodríguez-Gironés et al., 1996; 2001; Wright and Leonard, 2002; see e.g. Leonard et al., 2000; Neuenschwander et al., 2003; Kim et al., 2011 for empirical supports). In animal systems where lethal competition does not occur, two main forms of sibling rivalry can be identified: scramble competition and hierarchical competition. Scramble competition occurs among offspring with similar competitive ability and the individual success in receiving food is strictly dependent on own current begging level compared to brood mates. Hierarchical competition occurs whenever a strong dominance (e.g. mediated by offspring size and/or hatching asynchrony in birds) among offspring exists and the allocation of food is thus under the control of the most competitive individual(s) (Mock and Parker, 1997; Wright and Leonard, 2002; see also **Paragraphs 1.2, 1.3** and **1.6**). In the scramble model, begging strategies of individual offspring are predicted to vary according to competitive ability relative to siblings, to the balance between direct and indirect costs and benefits of subtracting food to kin, and to current level of need (see e.g. Rodríguez-Gironés et al., 1996; 2001; Price et al., 2002). Conversely, in the hierarchical model begging strategies should depend on the rank of individual offspring: smaller, marginal individuals (i.e. individuals with larger long-term need) generally beg more but obtain less food compared to dominant, larger brood mates which, in turn, can monopolize resources and modulate competitive effort in order to maximize their own fitness according to their relative satiation level compared to brood mates (Glassey and Forbes, 2002a; Price et al., 2002; but see Bonisoli-Alquati et al., 2011).

It is therefore widely acknowledged that individuals do modulate their begging according to the presence and/or behaviour of

brood mates. Despite an escalation of begging intensity is expected to occur in large broods (see e.g. Smith and Montgomerie, 1991; Kacelnik et al., 1995; Price, 1996; Neuenschwander et al., 2003; Kim et al., 2011), recent theoretical (Johnstone, 2004; Forbes, 2007) and experimental studies (Mathevon and Charrier, 2004; Bell, 2007; Madden et al., 2009; Marques et al., 2011) have pointed out that individual begging behaviour does not always vary in the direction of an increase in intensity in response to the rising begging levels of brood mates. An intriguing alternative is the so-called 'cooperative begging' (Johnstone, 2004): when begging functions as a constant stimulus to influence the total parental food provisioning, offspring may cooperate to maintain an overall level of begging. In such circumstances, any individual offspring is expected to decrease its begging intensity when begging levels of brood mates increase, resulting in an overall *per capita* lower begging intensity (see e.g. Mathevon and Carrier, 2004; Bell 2007; Madden et al., 2009).

1.5 Parent-absent begging

Besides addressing begging displays towards attending parents, offspring of several bird species have been observed to perform begging also when parents are not in the proximity of the nest (Clemmons, 1995; Budden and Wright, 2001; Leonard and Horn, 2001b; Leonard et al., 2005; Bulmer et al., 2008; Rivers et al., 2009; Roulin et al., 2009; Dreiss et al., 2010b). Since the so-called 'parent-absent begging' does not provide any evident benefit because it is not immediately linked to parental food provisioning while potentially increasing the costs of performing begging (Leonard and Horn, 2001b; Leonard et al., 2005), it has been considered as a misdirected behaviour, caused by low sensitivity in correctly detecting parental feeding visits (Leonard and Horn, 2001b; Leonard et al., 2005), especially in young nestlings (Budden and Wright, 2001; Dor et al., 2007; but see Leonard and Horn, 2001b). However, it has also been suggested that such behaviour can adaptively function to minimize the cost of missing parental feeding visits (Dor et al., 2007).

However, it has also been hypothesized that parent-absent begging serves to promote adaptive 'negotiation' among siblings about the optimal outcome of subsequent events of competition for parental resources (Sibling Negotiation Hypothesis: Roulin et al., 2000; Johnstone and Roulin, 2003; Roulin, 2004; Roulin et al., 2009; Dreiss et al., 2010b). Offspring might thus signal to each other their individual state and willingness to compete for the next indivisible food item delivered by parents. Therefore, by means of parent-absent begging, each individual can potentially assess the payoffs of sib-sib competition, and adopt optimal trade-offs between escalating begging intensity during the next feeding event(s) to outcompete nest mates and refraining from competing with needier siblings, since its own chances of being fed should be low (Roulin et al., 2000; Johnstone and Roulin, 2003; Roulin, 2004; Roulin et al., 2009; Dreiss et al., 2010b). Demonstration of the existence of a negotiation for parental resources among offspring has been provided only in one species, the barn owl (*Tyto alba*), while in other model systems evidence is lacking.

1.6 Factors affecting begging and phenotypic quality in nestling birds

When attending parents arrive to the nest with food, a cacophonous symphony of loud vocalizations, accompanied by the display of brightly coloured open gapes and intense, vigorous movements welcome them. Nestlings of many bird species in fact solicit parental care by simultaneously combining diverse visual and acoustic stimuli, thus making begging behaviour a very complex multi-component signal (e.g. Leonard et al., 2003; Kim et al., 2011; see a review in Kilner, 2002).

The evolution of different begging displays has been favoured because complex signals may improve the efficacy of communication and thus induce stronger responses (Rowe, 1999), or because they may provide receivers with reliable information about the condition of the signaller, particularly when every component carries information on different aspects of general state of the signaller (Johnstone, 1995; 1996; see Sacchi et al., 2001 for empirical support). In addition, multiple elements may provide redundant information, thus reducing perceptual errors, or be necessary to maintain reliable signalling throughout the entire nestling period (Kilner, 2002).

Experimental studies have provided evidence that single components of begging may have different functions in affecting parental food provisioning and allocation and may thus have evolved independently (Leonard et al., 2003; Kim et al., 2011). It has been suggested that acoustic displays (e.g. calls and vocalizations) influence food provisioning rate (Burford et al., 1998; Leonard and Horn, 2001c; Leonard et al., 2003; but see Clark and Lee, 1998), that postural displays (e.g. posturing and jostling) have an important role in determining the share of food among nestlings (Redondo and Castro, 1992; Kacelnik et al., 1995; Cotton et al., 1999), while chromatic traits (e.g. gape and flanges colour) seem to favour nestlings detectability in the nest (Ingram, 1920; Kilner and Davies, 1998; Avilés et al., 2008). However, different elements of begging may reciprocally interact in enhancing transmission of information about signallers, thus rendering the complex signal more profitable to be used by parents (Rowe, 1999; Glassey and Forbes, 2002b; see also Kilner, 2002). The intensity of visual (both gaping and posturing) and vocal components usually varies jointly according to nestling hunger, poor physical condition and begging of nest mates, indicating that different elements can act together in order to enforce parental response (Kilner et al., 1999; Glassey and Forbes, 2002b; Boncoraglio et al., 2009).

In birds, various factors, other than the number of competitors in the nest, have been demonstrated to potentially affect the expression of begging behaviour. On one hand, such factors can contribute to generate different categories of offspring in the brood (see **Paragraphs 1.2**, **1.3** and **1.4**) and thus to affect the within-brood competitive environment and the individual begging strategies (e.g. sex, hatching asynchrony, maternal effects; reviewed in Glassey and Forbes, 2002a). On the other hand, parental decisions over time and place of breeding can considerably influence the rearing environment (e.g. food abundance and quality, parasites, predators) to which all the offspring are exposed, possibly resulting in variation of individual phenotypic quality and, therefore, begging behaviour.

The importance of sex in determining offspring resource demands and/or daily energy expenditure, and sex-specific susceptibility to environmental conditions has been explained in the **Paragraph 1.3** (see also Hardy, 2002). Sex-related food demands by offspring may result in marked differences in the begging strategies by sons and daughters. Indeed, experimental studies have disclosed differences in begging behaviour by male and females nestlings (e.g. Saino et al., 2003b; 2008; Boncoraglio et al., 2008) and sex-related asymmetries in competitive abilities, that may ultimately cause a mortality bias towards the less competitive sex (Teather and Weatherhead, 1989; Arroyo et al., 2002; Fargallo et al., 2002; 2003).

Another important factor in moulding begging behaviour in birds, generating asymmetries in offspring phenotype, is hatching asynchrony (see e.g. Magrath, 1990; Stenning, 1996): in general, late-hatched nestlings are smaller and qualitatively inferior than early-hatched siblings (e.g. Saino et al., 2001; Müller et al., 2003; see also Glassey and Forbes, 2002a; **Paragraphs 1.2** and **1.3**). As also described in **Paragraph 1.4**, smaller offspring usually beg at higher intensity but suffer restricted access to limiting parental resources compared to their larger, competitively superior nest mates (e.g. Kacelnik et al., 1995; Cotton et al., 1999), unless parents have evolved strategies to counteract such asymmetry, by producing eggs increasing in size and allocating maternal effects according to laying order (Saino et al., 2001; Saino et al., 2002; Eising and Groothuis, 2003; Ferrari et al. 2006), or by preferentially feeding smaller individuals (Stamps et al., 1985; Bonisoli-Alquati et al., 2011). Nevertheless, late-hatched nestlings have typically inferior survival prospect, especially when ecological conditions are severe or when parasites are abundant (Spear and Nur, 1994; Cotton et al., 1999; Müller et al., 2003).

Individual competitive ability can also be markedly affected by the amount and quality of resources allocated by the mother to the eggs (Eising and Groothuis, 2003; von Engelhardt et al., 2006; Loiseau et al., 2008). Such maternal effects include hormones and other substances, like antioxidants and vitamins, and can have immediate consequences not only on offspring begging and aggressive behaviour (Eising and Groothuis, 2003; von Engelhardt et al., 2006; Loiseau et al., 2008), but also on early development, physiology, and viability (see e.g. de Ayala et al., 2006; reviews in Williams, 1994; Mousseau and Fox, 1998; Groothuis et al., 2005), with carry-over consequences on performances also later in life (Alonso-Alvarez et al., 2006; Rubolini et al., 2006; reviews in Mousseau and Fox, 1998; Lindström, 1999).

With reference to the environmental conditions that can affect begging strategies via effects on individual development and physiology, the most crucial factor is the availability of resources. A large number of studies has shown that several aspects of the nestling phenotype, including immuno-competence (Saino et al., 1997; Norris and Evans, 2000; Brzek and Konarwzesky, 2007), antioxidant protection (Alonso-Alvarez et al., 2007), metabolic rates and growth trajectories (Fargallo et al., 2002; Searcy et al., 2004), strictly depend on the amount and quality of food provided by parents, that in turn is mainly determined on the environmental availability of resources (see also **Paragraph 1.7**).

Finally, given their pervasiveness in natural environments, parasites are an additional important source of variation in nestling long-term need. A plethora of different ecto- and endo-parasites can attack birds especially during growth, when individuals are relatively naïve to exogenous antigens and pathogens, with several detrimental effects on development and survival (Tschirren et al., 2003; Fitze et al., 2004; Martinez-Padilla et al., 2004). In nestling birds, parasite burden can be mirrored by different components of begging: begging rate and chromatic traits, for example, have been suggested to reveal the exposure of nestlings to both ecto- (Christe et al., 1996) and endo-parasites (Saino et al., 2000a; 2000b).

1.7 Parental decisions over reproduction and long-term fitness consequences on offspring

The conditions experienced during early life, including parental care received and competition with brood mates, are therefore fundamental in determining individual developmental trajectories and survival to the independence from parents (see above). However, large differences in lifetime reproductive success among individuals, via variation in viability and/or breeding performance, can also depend on differences in the conditions under which growth from birth to sexual maturation take place (Lindström, 1999; Metcalfe and Monaghan, 2001; Ricklefs et al., 2006; Cam et al., 2011).

Rearing environment is mainly determined by differences in parental quality and environmental conditions, that in turn may partly depend on the phenotypic quality and the age of parents. Parental habitat selection affects the spatial environment in which offspring are reared. In addition, maternal choice of breeding date considerably influences the temporal environment in which the offspring will develop, and it is particularly important in seasonally varying environments where ecological conditions typically deteriorate as the breeding season progresses (see Dubiec and Cichoñ, 2001; Grüebler and Naef-Daenzer, 2008; Verhulst and Nilsson, 2008). Both spatial and temporal breeding decisions, combined with the parental investment towards the progeny (potentially skewed by competition among siblings; see above), thus determine the perinatal environment, because they affect the amount and the quality of food and the extent of maternal effects, as well as the presence of predators and parasites.

In birds, diverse environmental factors experienced in the nestling period have been related with phenotypic quality in adulthood, ultimately determining difference in lifespan, sexual attractiveness and breeding performance. Spatial and temporal variation of the natal site (Cam et al., 2003; van de Pol et al., 2006), as well as nutritional conditions (Birkhead et al., 1999; Blount et al., 2006; Naguib and Nemitz, 2007) and the occurrence of parasites (Eraud et al., 2009) during early life have been demonstrated to influence growth and viability, and then impact on adults in various ways. In addition, differential parental investment on individual members of the progeny, like the maternal effects allocated to eggs (Alonso-Alvarez et al., 2006; Naguib et al., 2006; Rubolini et al. 2006; Noguera et al., 2012) and the duration of parental care received (Cam et al., 2003), have been shown to have important carry-over effects until sexual maturity.

Outline of the study

The present study is divided into three parts. The first part (**Chapters 1-3**) is concerned with the consequences of intra-family communication in an altricial bird species, the barn swallow (*Hirundo rustica*), with special focus on the factors that can influence the expression of begging behaviour (e.g. sex, hunger level, immune challenge, level of need of nest mates), and thus mediate sibling competition and parental food allocation, and ultimately cause variation in offspring general state and growth trajectories.

In **Chapter 1**, I have examined begging behaviour by barn swallow nestlings in relation to siblings' need in order to investigate state-dependent expression of selfish/altruistic behaviour among siblings. I have compared begging behaviour and number of feedings received by dyads of nestlings in two different conditions: after both had been fed normally and after one of them had been food-deprived. The main aim of this study was therefore to evaluate whether nestlings in good condition reduced their competitive effort when facing a sibling after a period of food deficiency, thus favouring the access to food by needy kin and gaining indirect fitness benefits.

In **Chapter 2**, I have investigated the function of sib-sib interactions mediated by a peculiar form of within-brood communication that occurs in the absence of parents (the so-called parent-absent begging), both under normal and experimentally reduced food intake. In an experiment involving dyads of nestlings, I have particularly focused my attention on the effects of parent-absent begging on the competitive behaviour of interacting siblings and its consequences for access to food, possibly demonstrating a partial control on the allocation of parental investment by the offspring.

Chapter 3 deals with the sex-related effects of an immune challenge on body and feather growth as well as on the expression of morphological (i.e. palate and flanges coloration) and behavioural (i.e. postural intensity) begging displays involved in parent-offspring communication and competition among siblings. I have experimentally manipulated offspring condition by exposing the nestlings to an endotoxin extracted from bacterial cell walls (*Escherichia coli* lipopolysaccharide, LPS), aiming at evaluating whether begging displays, both under normal food provisioning regime and under food shortage, can convey reliable information about bacterial infection, and can affect the patterns of parental food allocation towards each individual member of the progeny.

The second part of the thesis (**Chapters 4-5**) is focused on variation of parental investment towards offspring of either sex according to spatial heterogeneity and seasonality of ecological conditions in two passerine birds, the barn swallow and the common starling (*Sturnus vulgaris*). In **Chapter 4**, I have examined variation in the proportion of male and female offspring fledged from first and second broods of the same barn swallow breeding pairs in relation to the nesting habitat quality. In particular, I have analyzed whether parents breeding in colonies in good ecological conditions (e.g. surrounded by large extent of hayfields, the main foraging habitat for this insectivorous bird) and with different demographic trend produced an unbalanced sex ratio consistent with adaptive sex allocation strategies, thus favouring the sex with smaller

natal dispersal and thus more likely to benefit from local environment.

Chapter 5 deals with seasonal variation in growth patterns, offspring quality and fledging prospects of male and female nestlings of the common starling. I have measured body mass, tarsus and feather growth during ontogeny as well as the antioxidant response to an immune challenge (LPS endotoxin) and hematocrit (i.e. proportion of red blood cells over total blood volume) in order to describe the between-sexes variation in nestling condition and mortality during the progression of the breeding season, potentially reflecting differential parental investment by parents or sex-related pattern of intra-brood hierarchy.

In the third part of the thesis (**Chapters 6-7**) I have considered the carry-over effects of rearing conditions on longevity and reproductive success in the short-lived barn swallow. More specifically, in **Chapter 6** I have investigated the role of hatching date, natal colony and individual condition relative to nest mates (e.g. immune response and body size), in determining lifespan and lifetime reproductive success (i.e. the number of nestlings successfully fledged).

Finally, **Chapter 7** is focused on preliminary analyses concerning potential long-term effects on breeding performance at sexual maturation (i.e. 1 year old) of a bacterial immune challenge during the early post-natal stage. I have compared the reproductive success at first breeding attempt of individuals exposed to a bacterial endotoxin (i.e. injection of lipopolysaccharide) when nestlings or to a control treatment (i.e. injection of phosphate-buffered saline solution) when recruited in the natal site as breeding adults.

To conclude, I provide a synthetic overview of the main findings which are discussed in detail in the text.

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PART I

BEGGING, SIBLING COMPETITION AND KIN SELECTION

Chapter 1

With a little help from my kin: barn swallow nestlings modulate solicitation of parental care according to nestmates' need

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With a little help from my kin: barn swallow nestlings modulate solicitation of parental care according to nestmates' need

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sibling interactions.

Abstract

In altricial species, offspring competing for access to limiting parental resources (e.g. food) are selected to achieve an optimal balance between the costs of scrambling for food, the benefits of being fed and the indirect costs of subtracting food to relatives. As the marginal benefits of acquiring additional food decrease with decreasing levels of need, satiated offspring should be prone to favour access to food by their needy kin, thus enhancing their own indirect fitness, while concomitantly reducing costs of harsh competition with hungry broodmates. We tested this prediction in feeding trials of barn swallow (*Hirundo rustica*) nestlings by comparing begging behaviour and food intake of two similar-sized nestmates, one of which was food-deprived (FD). Non-food-deprived (NFD) offspring modulated begging intensity depending on their nestmate's need: when competing with FD nestmates, NFD nestlings reduced both the intensity and frequency of begging displays compared to themselves in the control trial before food deprivation. Hence, NFD nestlings reduced their competitiveness to the advantage of FD nestmates, which obtained more feedings and showed a threefold larger increase in body mass. Moderation of individual selfishness can therefore be adaptive in the presence of a needier kin, because the indirect fitness benefits of promoting its condition can outweigh the costs of forgoing being fed, and because it limits the cost of begging escalation against a vigorous competitor.

Introduction

Altricial offspring from diverse taxa solicit provisioning of parental care via complex 'begging' displays (Kilner & Johnstone, 1997; Wright & Leonard, 2002). Theory posits that begging displays have evolved either as traits that offspring use to manipulate parental decisions over the distribution of care to their own advantage, or as signals of need that parents use to design allocation strategies that maximize their own Darwinian fitness (Trivers, 1974; Godfray, 1995; Kilner & Johnstone, 1997; Wright & Leonard, 2002). Many studies have provided evidence that begging levels increase with offspring hunger and affect food provisioning (e.g. Cotton *et al.*, 1996; Boncoraglio *et al.*, 2008; Dor & Lotem, 2011; for a review see Mock *et al.*, 2011). Moreover, theoretical studies predict that individual begging behaviour may change according to the presence and behaviour of competitors, depending on individual fitness pay-offs (Godfray, 1995; Johnstone, 2004). In case of scrambling competition for access to food, offspring may escalate begging intensity to obtain a greater share of limiting resources (Smith & Montgomerie, 1991; Godfray, 1995; Price, 1996). Offspring may also be selected to 'cooperate' in order to maintain a threshold begging intensity that elicits care provisioning by parents, potentially causing negative covariation between begging intensity of individual nestlings and overall begging intensity of their brood (Johnstone, 2004; Bell, 2007; Forbes, 2007).

Kin selection is expected to play a key role in the evolution of begging strategies (Godfray, 1995; Mock & Parker, 1997; Wright & Leonard, 2002; Moreno-Rueda, 2007): whenever the indirect fitness benefits exceed the direct fitness costs, offspring may reduce their selfishness

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(Hamilton, 1964). However, a kin selection perspective on the evolution of sibling interactions has been largely neglected in experimental studies of begging (Wright & Leonard, 2002; Forbes, 2007), despite the obvious indirect fitness costs of reducing the amount of food provisioned to kin (but see, e.g. Briskie *et al.*, 1994; Royle *et al.*, 1999; Boncoraglio & Saino, 2008; Boncoraglio *et al.*, 2009). Irrespective of kinship, the pay-off of acquiring an additional unit of food may decrease with decreasing need, leading to predict that less needy offspring may allow a larger share of food to be allocated to their hungry competitors (Godfray, 1995; Wright & Leonard, 2002; Forbes, 2007). Either mechanism may intervene independently or in concert in modulating individual selfishness in sib–sib interactions.

In this study, we experimentally investigated the consequences of variation in hunger level of barn swallow (*Hirundo rustica*) nestlings for begging behaviour and access to food by the interacting siblings. Our main focus was on the behaviour of a normally fed [i.e. non-food-deprived (NFD)] nestling in feeding trials under two contrasting experimental conditions: when competing with a nestmate of the same level of need [before food deprivation trial (BFD) hereafter], and when competing with the same, food-deprived (FD) nestmate [after food deprivation trial (AFD)]. Thus, for the NFD nestling, the individual fitness benefit of being fed was the same in the two trials, and we assessed how it responded to variation in hunger level of its FD nestmate.

We expected FD nestlings to increase begging intensity in the AFD vs. the BFD trial (Boncoraglio *et al.*, 2009; Bonisoli-Alquati *et al.*, 2011; Romano *et al.*, 2011). In the AFD trial, the NFD nestling could be expected to behave in three different ways. First, compared to the BFD trial, it might not modify its behaviour, which would indicate that begging signals are dependent on current need and independent of the competition context (Cotton *et al.*, 1996). Second, it could increase its begging level, which would indicate an escalation of competitive efforts (Smith & Montgomerie, 1991; Price, 1996). Finally, a third possibility is that the NFD nestling decreases its begging intensity. This is expected to occur whenever the indirect fitness gain of favouring a needy kin exceeds the direct benefit of receiving more food, which in turn will depend on the energetic costs of escalating sibling competition with a highly motivated nestmate and the marginal benefit of acquiring an additional food item, as well as on the actual genetic relatedness to the competitor in a species where broods can have mixed parentage.

Materials and methods

Study species and field procedures

The barn swallow is a small (ca. 20 g), migratory passerine with biparental care of altricial nestlings (Møller, 1994). Females lay 1–3 clutches of 3–7 eggs per breeding season and nestlings fledge when 19–21 days old (Møller, 1994). Parents normally feed a single nestling per visit to the nest (Møller, 1994). Previous studies have shown that begging intensity and parental provisioning increase with nestling hunger (Boncoraglio & Saino, 2008; Boncoraglio *et al.*, 2008; Romano *et al.*, 2011). Sibling competition increases when competitors are unrelated, suggesting nestlings' ability at discriminating kin (Boncoraglio & Saino, 2008; Boncoraglio *et al.*, 2009).

The study was carried out in 2011 (June to August) in three colonies near Milan (Northern Italy). Nests were visited daily to record breeding events. Feeding trials were conducted on consecutive days starting when nestlings were 9.62 ± 1.27 SD (range 7–11) days old. A few minutes before starting the first trial, we ringed all nestlings and measured their body mass (to the nearest 0.1 g) and tarsus length (to the nearest 0.01 mm). As nestling barn swallows reach their peak body mass by day 12 after hatching and body mass declines thereafter, nestlings were tested before their prefledging mass recession, implying that body mass reliably reflected the within-brood size/age hierarchy (Bonisoli-Alquati *et al.*, 2011). Parental food allocation and competitive interactions among nestmates may vary in relation to the age or size of the nestlings (Bonisoli-Alquati *et al.*, 2011): to reduce such possible confounding effects, in each brood we choose the dyad of nestlings with the smallest difference in mass to be tested in feeding trials (hereafter 'focal' nestlings). When more than one dyad of nestlings could be identified with this criterion, the choice of focal nestlings was randomized. Body mass of focal nestlings was larger than that of nestmates (paired $t_{21} = 2.19$, $P = 0.040$), indicating that smaller nestlings tended to be excluded from the trials. We preferred to focus on the behaviour of two nestlings, rather than of the entire brood, in order to adhere to theoretical models of sibling interactions (e.g. Godfray, 1995; Johnstone, 2004), which were developed for pairs of interacting offspring, and to simplify the behavioural observations.

To test for modulation in competitive interactions by individual nestlings, we compared postural begging and the number of feedings received by each nestling in two different conditions: after both had been fed normally (BFD) and after one of them had been food-deprived (AFD). We also recorded body mass change during the trials (=body mass at the end minus body mass at the start of the trial). Before food deprivation trials were always conducted the day before AFD trials to avoid any interference of food deprivation on within-brood hierarchies in the second trial. While this design had the important consequence that food deprivation did not affect BFD observations, variation in begging behaviour between the two trials might have partly arisen because of age or repeated testing effects.

However, these possibilities could be ruled out by re-analysing the data set of a previous experiment (Boncoraglio *et al.*, 2009). In fact, postural (intensity and duration of begging bouts) and vocal (loudness of calls, calling rate and number of syllables per begging bout) begging displays of nestlings of similar age as in the present study and tested under the same level of need did not vary over consecutive days (repeated measures mixed models, $n = 29$ nestlings; in all cases: $F_{1,27} < 2.43$; $P > 0.13$). In addition, we did not find any effect of age (9 vs. 10 days after hatching) on postural begging intensity of both nestlings in BFD trials (see Results).

Trials always started at 10:00–11:00 am. The two focal nestlings were individually marked and left in the nest for a 90-min BFD feeding trial, whereas the nonfocal ones were removed and kept in a warm cloth bag. At the end of the trial, the two focal nestlings were weighed again and their siblings were returned to the nest. The next day, soon before the trial started, the two focal nestlings were weighed, and one of them, randomly chosen (the FD nestling), was removed from the nest, whereas the other (the NFD nestling) was handled shortly and left in the nest with all the other siblings for a normal food provisioning period lasting 120 min. The FD nestling was then returned to the nest for a 90-min AFD feeding trial with the NFD nestling, whereas the nonfocal nestlings were removed from the nest. The 120-min food deprivation was intended to simulate a short period of starvation, similar to those naturally occurring during inclement weather, and led to a decrease in body mass of 0.82 g (0.05 SE). This decrease resulted in a 1.00 g (0.23 SE) (i.e. 5% of body mass of NFD nestlings) difference in body mass between FD and NFD nestlings before the start of the AFD trial.

Nestling behaviour during the trials was videotaped with a Sony DCR-SR72E digital camera located 2–4 m from the nest. The intensity of postural begging ('begging intensity') during each feeding visit by parents was scored on a four-level scale ranging from 0 (nestling stands motionless and does not beg when parents visit the nest) to 3 (nestling begs intensely while fully stretching the neck and legs towards the attending parent) (see Boncoraglio *et al.*, 2009 for details). The number of feedings was used as a proxy for the food share obtained by individual nestlings (Boncoraglio *et al.*, 2008). We calculated the proportion of feedings obtained by the NFD nestling in each trial (i.e. the ratio between the number of feedings obtained by the NFD nestling and the number of feedings delivered by parents to the nest). In addition, as feeding rate does not account for variation in quality and size of individual feedings, we also recorded change in body mass during the trials because it integrates both food intake and physiological costs of begging (e.g. Kilner *et al.*, 2004; Boncoraglio *et al.*, 2008, 2009): this measure may thus be considered as a proxy for food intake and the balance between benefits and costs of scrambling (see also Romano *et al.*, 2011).

Sibling interactions and begging behaviour could vary with time elapsed since the handling/start of trial (both trials) because of variation in the relative satiation levels of the nestlings (AFD trial only). We thus tested for temporal variation in begging intensity and in the competitive interactions won by nestlings. To this goal, we *a posteriori* divided each trial into three 'feeding periods', each including one-third of the feeding events recorded during that particular trial. We preferred to divide trials into periods based on the number of feedings rather than on time since the start of the trial because hunger, and thus begging behaviour, should depend more strictly on the amount of food received than on time elapsed since the start of the trial.

All the measurements were performed blind with respect to treatment. We videotaped 21 dyads of focal nestlings, while data on body mass change were available for 22 dyads.

Statistical analyses

Analyses were performed using repeated measures mixed models with the MIXED and GLIMMIX procedures of SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA). Feeding trial (BFD or AFD), nestling status (FD or NFD) and a three-level factor accounting for the feeding period were included in the models as fixed effects, together with their interactions. Dyad (i.e. nest) and nestling identity were included as random intercept effects.

Begging intensity was analysed using a multinomial mixed design (though for ease of interpretation, we report mean score values in the Results section). The models did not converge when we included the random effect of dyad, which was therefore removed (the results were similar in Gaussian linear mixed models (LMM) including dyad as a random effect; details not shown). The number of feedings was analysed using a Gaussian LMM. Variation in the proportion of feeding events for each nestling when it did not beg (number of events with begging score equal to 0 divided by the total number of feeding events; 'proportion of no-begging events' hereafter) and in the proportion of feedings obtained by the NFD nestling (number of feedings received by the NFD nestling divided by the total number of feeding events) was tested in binomial mixed models.

The change in body mass between the start and end of the trial (body mass change) was analysed using repeated measures LMM including feeding trial, nestling status and their interaction as factors, and nest and nestling identity as random intercepts.

Differences between groups were explored by *post hoc* tests (planned comparisons were used for the multinomial model). As calculation of degrees of freedom in

mixed models is not straightforward (Zuur *et al.*, 2009), they were conservatively set as equal to the number of nests.

Results

Variation in begging intensity and food intake

Before the start of the BFD trial, body mass and tarsus length of FD and NFD nestlings did not differ significantly (paired *t*-test: $|t| < 1.72$, $P > 0.10$). The number of feeding events ranged from 10 to 44 in BFD trials (mean: 23.52 ± 7.99 SD) and from 15 to 44 in AFD trials (26.48 ± 7.57 SD) and did not significantly differ between trials (paired *t*-test: $t_{20} = 1.49$; $P = 0.15$).

Variation in begging and food provisioning variables according to the combined effects of nestling status and feeding trial differed among the three feeding periods (hereafter 'period'), as indicated by the significant three-way interaction among nestling status, feeding trial and period (begging intensity: $F_{2,21} = 3.57$, $P = 0.046$; proportion of no-begging events: $F_{2,21} = 7.36$, $P = 0.004$; number of feedings: $F_{2,21} = 10.55$, $P < 0.001$; see Table S1 for full model details). Similarly, the proportion of feedings obtained by the NFD nestling differentially varied among periods in the AFD and BFD trials ($F_{2,21} = 5.84$, $P = 0.010$).

To better uncover the joint effects of nestling status and feeding trial on begging and food intake variables, we ran separate mixed models for each period (Table 1). Overall, these models showed that differential effects of feeding trial according to nestling status were attenuated from the first to the last period (Table 1, Fig. 1). In the first period, all variables were significantly affected by the interaction between nestling status and feeding trial (Table 1). Importantly, AFD, NFD nestlings begged less vigorously, did not beg for a greater proportion of times and obtained fewer feedings compared to themselves BFD (Fig. 1). The opposite pattern emerged for FD nestlings, who received more feedings than NFD siblings (Fig. 1). In the second period, the effect of the interaction between nestling status and feeding trial persisted for the number of feedings and, marginally nonsignificantly, for begging intensity, with begging of NFD nestlings becoming less intense AFD compared to BFD ($t_{21} = 3.76$, $P = 0.001$), while this was not the case for FD nestlings ($t_{21} = 1.58$, $P = 0.130$; Table 1; Fig. 1). However, the interaction effects in the second period were generally weaker than in the first one (Table 1, Fig. 1). Finally, in the third period, no significant effects of the nestling status by feeding trial interaction emerged (Table 1). Consistent with the patterns observed for other variables, the proportion of feedings obtained by the NFD nestling differed between trials in the first period only (Table 1, Fig. 1).

Table 1 Mixed models of the effects of nestling status (FD or non-FD), feeding trial (before or after food deprivation) and their interaction during feeding periods on begging intensity, proportion of no-begging events and relative number of feedings received. The effect of feeding trial on the proportion of feedings received by non-FD nestlings in each period is also reported (see Methods).

	Period 1		Period 2		Period 3	
	F	*P*	*F*	*P*	*F*	*P*
Begging intensity						
Nestling status	1.25	0.276	0.06	0.803	3.85	0.063
Feeding trial	0.05	0.830	10.36	0.004	10.61	0.004
Nestling status × feeding trial	16.94	< 0.001	4.25	0.052	0.14	0.710
Proportion of no-begging events						
Nestling status	5.89	0.024	0.01	0.920	2.57	0.124
Feeding trial	2.80	0.109	1.06	0.316	0.04	0.850
Nestling status × feeding trial	22.53	< 0.001	2.76	0.112	0.33	0.574
Number of feedings received						
Nestling status	5.18	0.033	0.15	0.698	0.17	0.684
Feeding trial	1.08	0.310	2.03	0.169	4.64	0.043
Nestling status × feeding trial	27.03	< 0.001	6.05	0.023	0.70	0.411
Proportion of feedings received by non-food-deprived nestlings						
Feeding trial	17.61	< 0.001	3.34	0.082	0.30	0.587

FD, food-deprived.

To account for the potential confounding effects of among-nest variation in sibling competition, which is expected to increase with the number of siblings competing with the NFD nestlings during food deprivation (Rodríguez-Gironés *et al.*, 2002), on begging and food intake variables, we repeated all analyses (separately for each period) including brood size as a covariate and tested the effects of three-way interaction among nestling status, feeding trial and brood size (or the two-way interaction between feeding trial and brood size on the proportion of feedings obtained by the NFD nestling). No significant effect of the three-way interaction emerged for any variable in any period (in all cases: $F_{1,21} < 2.06$; $P > 0.17$). Similarly, no significant effect of the two-way interaction between feeding trial and brood size on the proportion of feedings obtained by the NFD nestling was found in any period (in all cases: $F_{1,21} < 0.06$; $P > 0.81$).

Begging intensity in the BFD trial did not vary according to nestling age when including in a multinomial mixed model the effect of age as a fixed factor ($F_{4,21} = 2.05$; $P = 0.12$). In addition the difference in the mean begging intensity between BFD and AFD trials of both FD and NFD nestlings did not vary according to nestling age considering either the entire feeding trials (Kruskal-Wallis test: NFD nestling: $P = 0.63$; FD nestling: $P = 0.98$) or the first period only (NFD nestling: $P = 0.13$; FD nestling: $P = 0.74$).

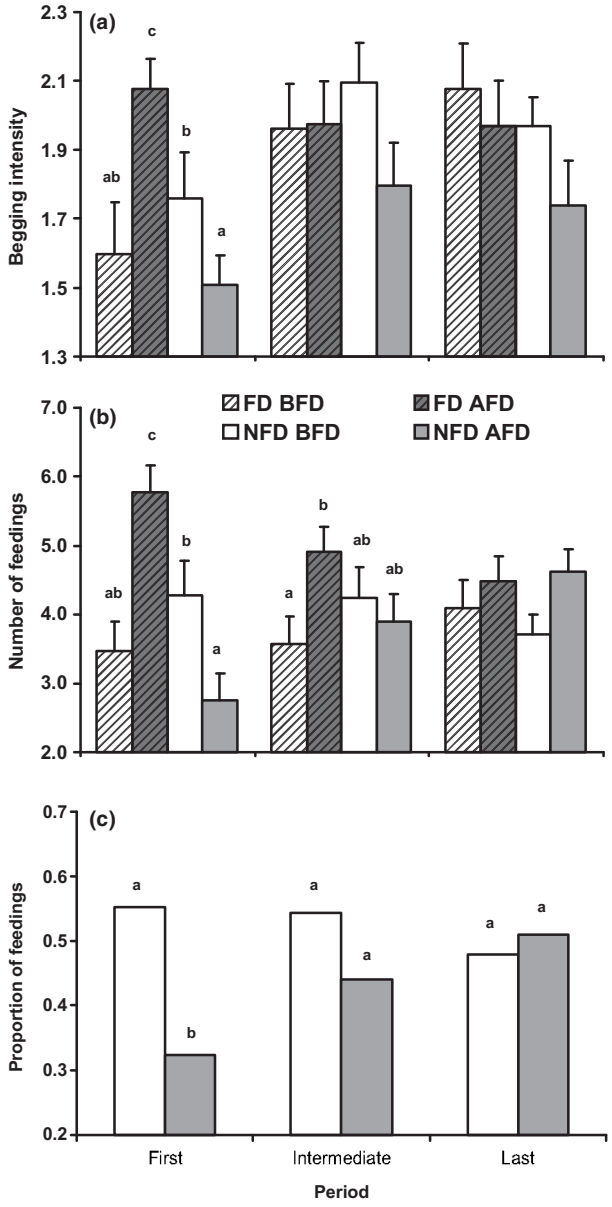

Fig. 1 Mean (+SE) (a) begging intensity and (b) number of feedings received by non-food-deprived (NFD) and food-deprived (FD) nestlings of 21 dyads during the feeding periods into which after food deprivation (AFD) and before food deprivation (BFD) trials were split; panel (c) shows the proportion of feedings obtained by NFD nestlings. Letters denote significant differences between groups within each period at *post hoc* (or planned comparisons tests for begging intensity) based on mixed models for each period; in (a) and (b), letters are not shown when the nestling status by feeding trial interaction was not statistically significant (see Table 1).

Change in body mass

Body mass change was affected by the interaction between feeding trial and nestling status ($F_{1,22} = 24.27$, $P < 0.001$): as expected, the FD nestling in the AFD trial gained more mass than its NFD sibling, whereas no significant differences between nestlings were observed in the BFD trial (Fig. 2). Mass gain of NFD nestlings did not differ between BFD and AFD trials (Fig. 2). However, in spite of the larger body mass gain, FD nestlings were still slightly lighter than their NFD siblings at the end of the AFD trial (19.47 g ± 0.36 SE vs. 19.92 g ± 0.38 SE; paired $t_{21} = 2.11$, $P = 0.047$). Finally, the summed change in body mass of both nestlings (i.e. the sum of body mass change of NFD and FD nestlings for each nest and trial) was significantly larger AFD than BFD (0.95 g ± 0.16 SE vs. 0.26 g ± 0.14 SE; paired $t_{21} = 3.97$, $P = 0.001$).

Discussion

We showed that nestling barn swallows modulate begging behaviour in response to hunger level of their nestmates: when confronted with FD nestmates, NFD nestlings reduced the intensity and frequency of begging displays and obtained fewer feedings compared to themselves when competing with the same nestling before it was FD. These effects emerged early in the AFD trial and vanished thereafter, likely because of the increasing hunger level of NFD nestlings and increasing satiation of FD nestmates.

Our findings argue against the hypotheses that begging intensity is independent of broodmates' need (Cotton *et al.*, 1996) and that NFD nestlings escalate begging to outcompete needy nestmates (see also Bonisoli-Alquati *et al.*, 2011). Rather, the results support the idea that NFD nestlings moderated their selfishness when exposed to hungry nestmates by reducing their competitive efforts in the AFD vs. BFD trial, thereby favouring access to food by needy FD nestmates. We regard the possibility that the behaviour of NFD

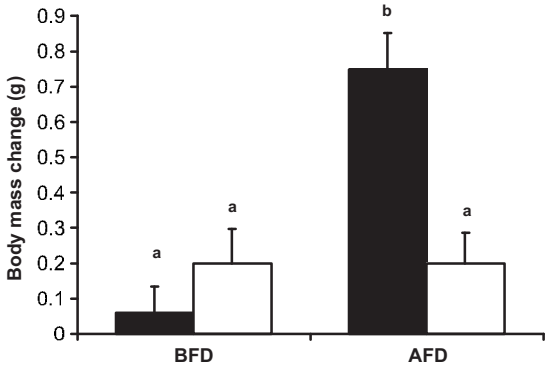

Fig. 2 Mean (+SE) body mass change before (BFD) and after (AFD) a period of food deprivation in 21 dyads of nestlings. Black bars: food-deprived nestlings; white bars: non-food-deprived nestlings. Different letters indicate differences between groups at *post hoc* tests.

nestlings was caused by a direct interference by FD nestmates, which may, for example, prevent them to move towards attending parents, as an unlikely one as in open barn swallow nest cups there are no positions that are preferentially used by parents to feed the nestlings, in contrast to hole-nesting species (Cotton *et al.*, 1999).

Kin selection theory envisages that an 'altruistic' behaviour should be favoured if the direct fitness cost it entails (less food received) is at least compensated by an indirect fitness gain (more food obtained by a sibling in poor condition). Our findings show that FD nestlings benefitted from reduction in competitiveness by their nestmates, as they received more feedings and gained considerably more mass AFD than BFD. In the barn swallow, broodmates are closely related to each other (98% of nestlings are either full-sibs or half-sibs; see Boncoraglio & Saino, 2008). Moreover, previous studies demonstrated that barn swallow nestlings regulate their competitiveness according to kinship, also suggesting their ability in recognizing kin (Boncoraglio & Saino, 2008; Boncoraglio *et al.*, 2009). NFD nestlings may thus indirectly benefit by allowing their needy kin to recover from a food deficiency and enhancing their prefledging survival prospects. The apparent favouritism of NFD nestlings towards their kin did not entail obvious costs in terms of body mass change: indeed, NFD nestlings did not show any significant difference in body mass gain in the BFD vs. AFD trial, although it is possible that the decreased competitiveness by the NFD nestling resulted in lowered mass gain during the first part of the AFD trial, as a consequence of the small number of feeding received.

Irrespective of the degree of relatedness with their counterpart, modulation of competitiveness by NFD nestlings could be also consistent with a begging strategy that maximizes direct individual fitness by balancing the benefit of reducing energy expenditure of scramble competition against a hungry nestmate and the cost of missing feeding events. Nestlings may indeed communicate to each other their hunger level and willingness to compete for food delivered by parents: the more satiated individual may thus give up competing for the next food item, because the chances of being fed may decrease when nestmates are more hungry and motivated to scramble for it (Roulin *et al.*, 2000, 2009; Johnstone & Roulin, 2003).

In the present study, we cannot fully disentangle whether the decrease in begging intensity was caused by a kin-selected moderation of selfishness or by a reduction in the direct costs of scrambling competition for food only to maximize their direct fitness: however, we regard the latter possibility as less likely, because indirect fitness benefits derived from kin selection markedly affect the modulation of individual begging behaviour in this species, as nestlings beg more vigorously when the competitors are unrelated (Boncoraglio & Saino, 2008; Boncoraglio *et al.*, 2009). Nevertheless, we emphasize that both mechanisms may act in concert in moulding individual begging strategies and that a moderation of selfishness by nestlings in the presence of needier kin may be adaptive, by increasing indirect fitness, because nestmates clearly benefit from such reduced competitiveness, and avoiding the high cost of harsh competition against hungry nestmates. Additional studies, comparing begging behaviour in dyads of full-sibs, half-sibs or unrelated nestlings, will better clarify this scenario.

Surprisingly, despite the similar feeding rates in the two trials, the sum of mass gains by the two nestlings was 3.7 times larger in the AFD than in the BFD trial, entirely due to the larger body mass increase of FD nestlings. We might envisage two nonmutually exclusive explanations for this pattern. First, the behaviour of NFD nestlings during the AFD trial apparently reduced the intensity of scramble competition for food compared to the BFD trial, resulting in decreased costs of begging solicitations. As begging is an energetically costly behaviour (Kilner, 2001; Moreno-Rueda & Redondo, 2011; but see Martín-Galvez *et al.*, 2011), and given that individuals have limited amounts of resources to be allocated to different body functions (Zera & Harshmann, 2001), nestlings may have allocated more resources to other fitness-related traits, such as the maintenance of lipids stores and growth. Indeed, overall begging intensity was lower in the AFD trial (see Supporting information). Alternatively, parents may have increased the size or nutritional value of food items delivered. We can also safely rule out that a larger increase in body mass by FD nestlings in the AFD trial was solely caused by an 'empty gut' effect after the period of starvation, which could result in a lower defecation rate and in a subsequent larger mass gain per unit of food obtained. In fact, body mass change per unit of food ingested did not differ between FD and NFD nestlings during the AFD trial. Moreover, the difference in mass gain between nestlings in the AFD trial disappeared after controlling for the number of feedings received, implying that the larger change in body mass of FD nestlings was almost entirely due to the number of feedings received rather than to a different defecation rate compared to NFD nestmates (see Supporting information).

Only few previous studies demonstrated modulation of begging behaviour in response to siblings that is compatible with our findings. For example, tree swallow (*Tachycineta bicolor*) nestlings reduced calling duration when experimentally subjected to simulated high-intensity compared to low-intensity playback of nestmates' begging (Marques *et al.*, 2011): however, the above study did not investigate the actual variation in food intake and body mass gain deriving from such modulation. Perhaps, the most striking evidence of sibling altruism in nestling birds was observed on captive budgerigars, where well-fed first-hatched (senior) nestlings were sporadically observed to actively regurgi-

tate food to their needy last-hatched (junior) nestmates (Stamps *et al.*, 1985). In the barn swallow, previous studies of nestling begging behaviour provided suggestive evidence of a possible cooperative altruism among nestlings (Bonisoli-Alquati *et al.*, 2011). For example, smaller junior nestlings were not outcompeted by larger seniors both when normally fed and after a period of food deprivation (Bonisoli-Alquati *et al.*, 2011), but such studies did not directly test the variation of begging behaviour of nestlings in response to different levels of need by siblings.

In conclusion, we showed that barn swallow nestlings curtail their request of parental resources when competing with needy nestmates, thereby enhancing access of disadvantaged kin to parental care. These results seem best interpreted in the light of kin selection theory, as they suggest that individual nestlings can take advantage of a reduction in their selfishness in the presence of needy siblings because the indirect fitness benefits of promoting condition of kin outweigh the costs of forgoing being fed.

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Supporting information

Additional Supporting Information may be found in the online version of this article:
Table S1 Mixed models reporting the effects of nestling status (FD or NFD), feeding trial (BFD or AFD), interval and their two and three-way interactions on begging intensity, proportion of no-begging events, and number of feedings received.

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SUPPORTING INFORMATION

Table S1. Mixed models reporting the effects of nestling status (FD or NFD), feeding trial (BFD or AFD), interval and their two- and three-way interactions on begging intensity, proportion of no-begging events, and number of feedings received. Table also reports the effect of feeding trial, interval and their interaction on the proportion of feedings received by NFD nestlings. See Methods for details on mixed modelling procedures.

	F	*df*	*P*
Begging intensity			
Nestling status	1.07	1, 21	0.313
Feeding trial	11.93	1, 21	0.002
Interval	11.28	2, 21	< 0.001
Nestling status × Feeding trial	14.16	1, 21	0.001
Nestling status × Interval	0.71	2, 21	0.505
Interval × Feeding trial	5.55	2, 21	0.012
Nestling status × Feeding trial × Interval	3.57	2, 21	0.046
Proportion of no-begging events			
Nestling status	6.71	1, 21	0.017
Feeding trial	0.00	1, 21	0.955
Interval	1.81	2, 21	0.187
Nestling status × Feeding trial	10.24	1, 21	0.004
Nestling status × Interval	2.84	2, 21	0.081
Interval × Feeding trial	1.46	2, 21	0.254
Nestling status × Feeding trial × Interval	6.20	2, 21	0.008
Number of feedings received			
Nestling status	4.90	1, 21	0.038
Feeding trial	7.16	1, 21	0.014
Interval	0.22	2, 21	0.803
Nestling status × Feeding trial	19.03	1, 21	< 0.001
Nestling status × Interval	2.80	2, 21	0.083
Interval × Feeding trial	0.16	2, 21	0.854
Nestling status × Feeding trial × Interval	10.86	2, 21	< 0.001
Proportion of feedings received by NFD nestlings			
Feeding trial	10.60	1, 21	0.004
Interval	1.51	2, 21	0.245
Interval × Feeding trial	6.01	2, 21	0.009

Variation in begging intensity between the BFD and AFD trials

We analyzed the variation in the frequency of each category of begging score between the BFD and the AFD trial. Overall, the frequency of the different score categories differed between trials (multinomial mixed model with nestling identity as a random effect and feeding trial as a fixed factor, $F_{1,21} = 10.73$, $P = 0.004$). Specifically, the frequency of highest begging score (score 3) was larger in the BFD (43.9% of BFD begging events) compared to the AFD trial (33.2% of AFD begging events), whereas the frequency of score 2 was larger in AFD vs. BFD trial (37.6% vs. 29.9%). The frequency of begging scores 0 and 1 did not markedly differ between BFD and AFD trials (19.2% vs. 19.8% and 7.0% vs. 9.4%, respectively).

Body mass variation in relation to feedings received during the AFD trial

We evaluated whether the larger increase in body mass by the FD nestling in the AFD trial was caused by an 'empty gut' effect, implying a reduced defecation rate after the brief period of starvation: in this case, we might expect a larger mass gain per unit of food ingested by the FD nestlings compared to the NFD ones during the AFD trial. However, the slope of the relationship between mass variation and feedings received did not significantly vary according to nestling status (effect of the interaction between nestling status and number of feedings received, mixed model: $F_{1,21} = 1.36$, $P = 0.25$; model-estimated slopes: FD nestlings, 0.06 ± 0.02 SE, $t_{21} = 3.82$, $P = 0.001$; NFD nestlings, 0.04 ± 0.02 SE, $t_{21} = 2.51$, $P = 0.020$). Moreover, the main effect of nestling status was far from statistically significant ($F_{1,21} = 0.01$, $P = 0.93$), implying that the larger body mass gain by FD nestlings in the AFD trial disappeared when controlling for the number of feedings received. Thus, the larger mass gain by the FD nestling in the AFD trial was entirely due to the number of feedings received rather than different defecation rate compared to NFD nestmates.

Chapter 2

Parent-absent reliable signalling of need and its consequences for sibling competition in the barn swallow

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ABSTRACT

In species with parental care, competition among siblings for access to limiting parental resources is common. Sibling competition can be mediated by begging displays, which are addressed to the parents upon care provisioning, but can also be performed in the absence of the attending parents. This so-called parent-absent begging (PAB) may potentially affect intra-brood competition dynamics for access to food. We investigated the role of PAB on sibling interactions and its effect on food intake in barn swallow (*Hirundo rustica*) nestlings both under normal and experimentally reduced food intake. Frequency of PAB increased after food deprivation. Nestlings that had performed PAB increased their begging intensity upon the subsequent parental feeding visit, while siblings reduced their own begging level, but only when they had not been food-deprived. As a consequence, nestlings which had performed PAB before parental arrival had larger chances of receiving food. However, nestlings did not benefit from displaying PAB when competing with food-deprived siblings. Our findings indicate that PAB reliably reflects need of food and willingness to vigorously compete for the subsequent food item. By exploiting siblings' PAB displays, nestlings may optimally balance the costs of scrambling competition, the direct fitness gains of being fed and the indirect fitness costs of subtracting food to needy kin. However, large asymmetries in satiation between competitors may lead individual offspring to monopolize parental resources, irrespective of PAB displays.

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INTRODUCTION

Life-history strategies that maximize individual fitness may differ among family members (Clutton-Brock, 1991; Stearns, 1992). In species with parental care, the *per capita* share of resources received by an individual offspring progressively declines with increasing family size (Mock and Parker, 1997). Whenever the overall food demand by the progeny exceeds the availability of parental resources, conflicts of interest and competition among siblings over access to limited resources are expected to occur.

Sibling competition can be severely detrimental to part of the progeny, either because of physical aggression or starvation of younger/weaker offspring, whose access to parental resources is restrained by more competitive siblings (Wright and Leonard, 2002). In several taxa solicitation of care provisioning and competitive interactions among the offspring are mediated by begging displays (Kilner and Johnstone, 1997; Wright and Leonard, 2002). In birds, 'begging' may include diverse behavioural and morphological displays addressed by the young to their parents to solicit provisioning of care (Kilner and Johnstone, 1997; Wright and Leonard, 2002). The function of begging in the resolution of sibling conflicts is still vividly debated (e.g. Godfray, 1991;1995; Johnstone,

1999; Mock et al., 2011): irrespective of considering begging as an honest signal allowing parents to identify the neediest offspring in the brood (Godfray 1991; 1995), or as a means by which siblings gain access to parental resources via scrambling interactions (Macnair and Parker, 1979; Parker and Macnair, 1979), experimental studies have consistently demonstrated that higher begging intensity secures individual offspring a larger share of resources (reviewed in Mock et al., 2011), which in turn increases their direct fitness. However, such benefits are expected to decrease with increasing individual condition (e.g. Godfray, 1991). Moreover, higher begging intensity entails the offspring with larger costs of displaying (e.g. physiological costs: Boncoraglio et al., 2012) and potentially increases the starvation risk of outcompeted siblings (e.g. Cotton et al., 1999). Thus, offspring are selected to strike the optimal balance between selfish exploitation of parental resources and altruism towards kin according to variation in their own condition relative to that of the competing siblings (Romano et al., 2012).

Besides begging towards attending parents, offspring of several bird species perform begging also when parents are not present, the so-called 'parent-absent begging' (PAB). Being not immediately connected to parental food provisioning, PAB has been regarded as a misdirected behaviour, possibly functioning to minimize costly misses of parental feeding visits by needy nestlings (Budden and Wright, 2001; Leonard and Horn, 2001; Leonard et al., 2005; Dor et al., 2007). An intriguing alternative interpretation envisages PAB as a means that siblings adopt to negotiate access to parental resources at subsequent provisioning events (Roulin et al., 2000; Johnstone and Roulin, 2003; Roulin, 2004; Roulin et al., 2009; Dreiss et al., 2010). According to this 'Sibling Negotiation Hypothesis' (Roulin et al., 2000; Johnstone and Roulin, 2003), offspring might signal to each other their individual state and willingness to compete for the next indivisible food item and reciprocally modulate competition effort accordingly, in order to maximize individual inclusive fitness (Roulin et al., 2000; Johnstone and Roulin, 2003; Roulin et al., 2009; Dreiss et al., 2010).

We investigated the effects of spontaneous occurrences of PAB on sibling begging upon parental food provisioning and food intake in altricial nestlings of the barn swallow (*Hirundo rustica*), an insectivorous passerine with biparental care. In this species, parents feed a single offspring at each provisioning visit to the nest, and access to food cannot be monopolized by individual nestlings by e.g. occupying advantageous positions in the nest (pers. obs.), as conversely occurs in cavity-nesting species (e.g. Cotton et al., 1999). Sib-sib competition for access to food is mediated by the intensity of calling, gaping and posturing upon food provisioning visits (Boncoraglio et al., 2009; Romano et al., 2011; 2012), but nestlings also perform PAB between feeding visits, with ca. 30% of feeding visits preceded by PAB events in full broods (our unpubl. data). Begging intensity increases with hunger, and parents preferentially feed the nestlings that beg the most (Boncoraglio et al., 2009; Bonisoli-Alquati et al., 2011; Romano et al., 2011). Nestlings adjust their competitive behaviour according to the payoffs of increasing begging intensity to obtain additional food versus drawing back and

favouring a needy sibling (Romano et al, 2012).

We performed feeding trials whereby pairs of similar-sized nestlings were first tested under a normal food provisioning regime. The same pairs of nestlings were then tested under different satiation conditions, by temporarily food-depriving one of them before the start of the trial. We first tested whether PAB reliably reflects offspring need, as observed in other species (Roulin et al., 2001; Bulmer et al., 2008; Rivers, 2009). If this is the case, PAB rate should increase with increasing hunger level, and begging by individual nestlings upon parental visits should be more intense if they had previously performed PAB. Secondly, we examined whether nestlings respond to PAB of their siblings by reducing begging intensity during the subsequent feeding event, which is expected if nestlings modulate begging efforts in response to their siblings' contingent need (Romano et al., 2012). As a consequence, nestlings should benefit from performing PAB by increasing their food share at the very next provisioning visit. Finally, in line with the above reasoning, we might expect food-deprived nestlings to be less sensitive to PAB by non-food-deprived siblings, i.e. they may not decrease begging intensity at parental feeding visits when their non-food-deprived nestmates perform PAB.

MATERIAL AND METHODS

Experimental procedures and behavioural observations

This study was carried out between June and August 2011 in two colonies (= farms) located near Milano (Northern Italy). Nests were visited every second day to record breeding events. At 7-11 days of age (day 0 = day of hatching of the first egg) we ringed all nestlings and measured their body mass (nearest 0.1 g). The two nestmates that had the smallest difference in body mass ('focal nestlings') were selected to be tested in feeding trials, which started on the day of measurement. Trials were started when nestlings were 7 (n = 1 nest), 8 (n = 5), 9 (n = 2), 10 (n = 8), or 11 (n = 6) days old. When three or more nestmates had the same difference in body mass, the choice was randomized. Hence, nestlings with similar competitive ability as mediated by size were chosen (see Bonisoli-Alquati et al., 2011).

We videotaped the same pair of nestlings during two feeding trials. Before the first trial, both nestlings had been fed under a normal provisioning regime (hereafter, 'before food deprivation' trial, BFD); before the second trial (hereafter, 'after food deprivation' trial, AFD), either, randomly chosen, nestling was food-deprived for 120 min by removing it from the nest. To avoid any potential effect of food deprivation on body mass and hierarchy among nestmates, AFD trials were always performed the day after BFD trials.

Feeding trials started between 10:00 and 11:00 A.M., and were conducted as follows: focal nestlings were made individually recognizable by temporarily marking them with two small white spots on their head and left in the nest for 90 min to compete for food. Non-focal nestlings were removed from the nest and kept in a safe place at ambient temperature. After the trial, all nestlings were returned to the nest. The next day, one randomly-chosen focal nestling was removed from the nest for a 120

min period of food deprivation (food-deprived nestling). The other focal nestling (non-food-deprived nestling) was handled shortly, and left in the nest with all its siblings (the mean brood size of nests included in the study was 4.2 nestlings, range 3-6) for normal food provisioning. Food deprivation was intended to simulate a short period of starvation, as it may naturally occur in case of bad weather. The food-deprived nestling was then returned to the nest for the 90 min AFD trial together with the non-food-deprived one, while the non-focal nestlings were temporarily removed.

Dyads of nestlings were videotaped with a Sony DCR-SR72E camera, positioned at 2-4 m from the nest. For the purpose of present study, we recorded all the events of PAB, defined as any occurrence of postural begging in the absence of parents on the nest. Postural begging intensity of both focal nestlings at the first feeding visit by parents following each PAB by either nestling, as well as at feeding events not preceded by PAB, was evaluated using a four-levels scale of postural scores (0 = no begging; 1 = gape open; 2 = gape open and neck stretched; 3 = vigorous begging with full stretched neck and tarsi). In the barn swallow, postural and vocal begging are strictly related (our unpubl. data): we are thus confident that postural begging score should reliably reflect the overall intensity of begging displays. We limited our analyses to the first feeding event following a PAB because preliminary analyses revealed that PAB did not predict nestling behaviour and food intake during subsequent feeding visits (details not shown). Begging intensity in feeding events not preceded by PAB of either nestling was used as a measure of the 'baseline begging intensity'. We then recorded which nestling received the unit of food from parents during each feeding event.

To avoid the dissipation of the effect of food deprivation on begging intensity, for the AFD trial the analyses of begging intensity at parental arrival and food intake were restricted to the initial third of all feeding events of the trial, including on average 8.40 ± 2.55 SD (range 5-14) feedings (feeding rate in the BFD trial was 15.12 ± 5.77 SD feedings/h). We used data for the first third of feeding events because hunger, and thus begging behaviour, is expected to depend more strictly on the amount of food received rather than on time elapsed since the start of the trial (see also Romano et al., 2012). However, all results were qualitatively unaltered when we selected data for the first 30 min of the AFD trial (details not shown for brevity).

All the measurements on video recordings were performed blindly with respect to the experimental treatment. We recorded 22 pairs of nestlings that performed a total of 96 or 153 PAB in BFD and AFD trials, respectively. In nestling pairs, PAB constituted ca. 11.0% of total begging events (i.e. begging scores at parental arrival = 0 were excluded), and may occur in bouts (25% of PAB), consisting of 2-7 consecutive distinct events of PAB. There was no difference in the mean number of PAB per bout between food-deprived and non-food-deprived nestlings (details not shown). Moreover, there was no difference in mean begging intensity, nor in the probability of obtaining a feeding, after a single or multiple PAB (details not shown for brevity). For simplicity, in all the analyses of begging intensity at parental arrival and probability of obtaining a feeding, we therefore considered bouts of PAB as single PAB displays. Finally,

begging events at parental arrival preceded by simultaneous PAB by both nestlings were excluded from the analyses of begging intensity at parental arrival and probability of obtaining a feeding. This procedure resulted in a total of 39 or 31 feeding events preceded by a PAB of either nestling (i.e. ca. 10% of total feeding events, compared with ca. 30% in full broods (our unpubl. data)) in BFD and AFD trials, respectively, that were entered in the analyses of begging intensity upon parental feeding visits and food intake.

Statistical analyses

We relied on repeated-measures mixed models including pair (nest) and nestling identity as random intercept effects, using data from both feeding trials. However, to investigate the effect of PAB on nestling behaviour and outcome of sibling competition under normal food provisioning regime, some models were run on data from the BFD trial only (see Results). In these models, we only included nest identity as a random intercept effect.

Variation in the total number of PAB events per nestling during the 90 min feeding trials was analysed in Poisson models where feeding trial (BFD or AFD), experimental group (food-deprived or non-food-deprived nestling), and their interaction were included as fixed effects.

Variation in begging intensity at feeding events following own or sibling's PAB was analysed in a multinomial mixed model (though in the Results we show mean score values for ease of interpretation) including feeding trial and experimental group as fixed effects and a three-level factor (PAB category) indicating if the feeding event was: 1) preceded by own PAB, 2) preceded by a sibling's PAB or 3) not preceded by any PAB. Two- and three-way interactions between fixed effects were also included in the model. All the above analyses were repeated including age of nestlings as a covariate (see Budden et al., 2001), but its effect was always far from significance (*P*-values always > 0.43) and was therefore removed from the models (details not shown for brevity).

In addition, we tested the effects of PAB on the probability that a nestling did not beg at parental visits, by running a binomial mixed model including experimental group, a two-level factor indicating whether a feeding event was preceded (coded as 1) or not (coded as 0) by a PAB event by the sibling (i.e. excluding own PAB events), and their interaction as fixed effects.

Finally, we investigated whether the probability of obtaining a feeding after an own PAB varied according to experimental group, feeding trial and their interaction in binomial mixed models. We note that this analysis was carried out only for the nestlings that performed PAB, as the probability of the other nestling obtaining food was simply (1 – the probability of obtaining a food item after an own PAB).

In Poisson and multinomial mixed models, degrees of freedom were conservatively set equal to the number of pairs included in each analysis. Due to the unbalanced sample sizes for each level of the random effect and the uncertainties in estimating degrees of freedom in non-Gaussian mixed models, tests of fixed effects of binomial models were performed with *z*-scores. Mixed models were run with the SAS 9.1 GLIMMIX procedure (Poisson and multinomial models; SAS Institute, 2006) and the R 2.8.1 *lmer* procedure (binomial models;

Bates et al., 2008; R Developmental Core Team, 2008).

RESULTS

Effects of food deprivation on PAB

Across both feeding trials, the number of PAB events was affected by food deprivation, as shown by the effect of experimental group by feeding trial interaction ($F_{1,22}$ = 11.18, P = 0.003). As expected, in the BFD trial, the nestlings that were subsequently assigned to either treatment performed a similar number of PAB events, while in the AFD trial, the mean number of PAB events was higher for the food-deprived nestling, either compared to its non-food-deprived nestmate or to itself BFD (**Figure 1**). Conversely, the number of PAB by non-food-deprived nestlings did not change between the BFD and the AFD trials (**Figure 1**).

Effects of PAB and food deprivation on begging intensity at parental visits

In the BFD trial, begging intensity varied according to PAB category ($F_{2,22}$ = 6.02, P = 0.008). Specifically, nestlings begged at highest intensity at feeding events preceded by own PAB, at lowest intensity at feeding events preceded by their sibling's PAB, while begging intensity at feeding events not preceded by PAB was intermediate (**Figure 2**, all pairwise differences statistically significant at $P < 0.05$).

In a model including data of both trials, there was a statistically significant three-way interaction effect among experimental group, feeding trial and PAB category on begging intensity ($F_{2,22}$ = 5.03, P = 0.016). To better interpret this effect, we ran separate mixed

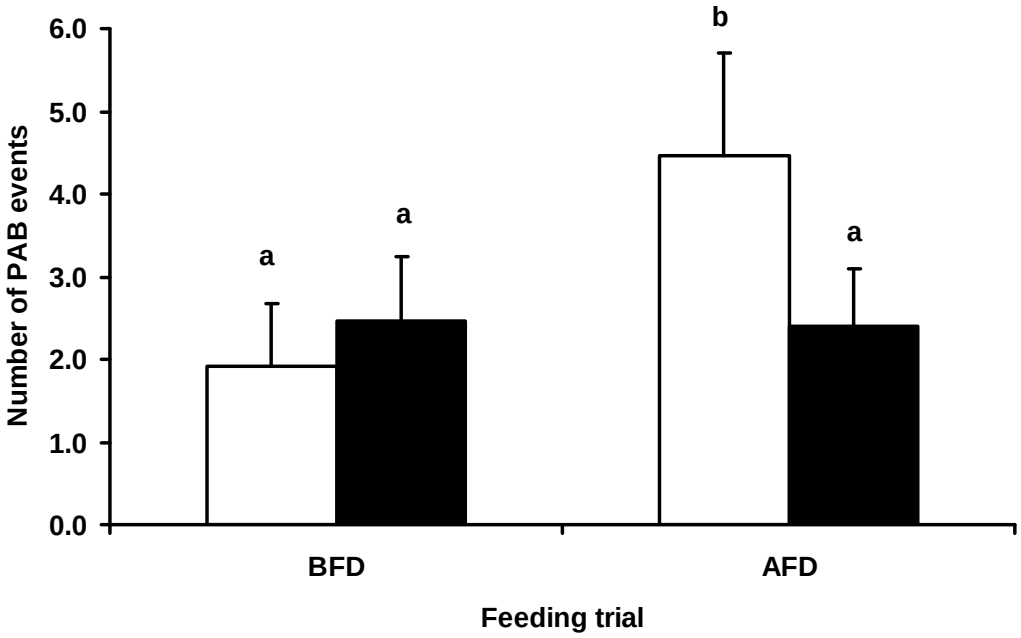

Figure 1. Mean (+ SE) number of parent-absent begging (PAB) events in 22 pairs of food-deprived (FD, white bars) and non-food-deprived (NFD, black bars) siblings before (BFD) or after (AFD) a period of food deprivation in 90 min feeding trials. Different letters indicate statistically significant differences ($P < 0.05$) between groups at *post-hoc* tests.

models for each feeding trial and explored the effects of the experimental group by PAB category interaction on begging intensity (**Table 1**). As expected because of random composition of experimental groups, in BFD trial the interaction effect was non-significant, whereas it was significant in the AFD trial (**Table 1**; **Figure 2**).

In the AFD trial, non-food-deprived nestlings behaved similarly to the BFD trial (**Figure 2**). However, begging intensity of food-deprived nestlings did not differ after their own compared to their sibling's PAB (**Figure 2**). Moreover, in feeding events not preceded by any PAB, begging intensity of food-deprived nestlings was significantly lower compared with feeding events following own or sibling's PAB (**Figure 2**).

In the BFD trial, the probability that a nestling did not beg (begging score = 0) increased at feeding visits preceded by a sibling's PAB compared to feeding visits not preceded by any sibling's PAB (1.047 ± 0.454 SE, $Z = 2.30$, $P = 0.021$), while the other model terms were non-significant (both $P > 0.75$). This analysis could be performed only on data for the BFD trial because models ran either on data from both trials (with feeding trial as a fixed factor) or on data from the AFD trial only did not converge, both including or excluding random effects (details not shown). ran either on data from both trials (with feeding trial as a fixed factor) or on data from the AFD trial only did not converge, both including or excluding random effects (details not shown).

Effects of PAB and food deprivation on food intake

In BFD trials, the probability of obtaining a unit of food in the first parental feeding visit following an own PAB was significantly larger (0.79) than expected by chance (0.50) (null binomial mixed model: $Z = 3.32$, $P < 0.001$; **Figure 3**).

In addition, the experimental group by feeding trial interaction effect on the probability of obtaining the first feeding after a PAB was marginally non-significant ($Z = 1.87$, $P = 0.061$). However, when trials were analysed separately, the probability of being fed after an own PAB was significantly larger for food-deprived nestlings than for non-food-deprived ones in the AFD trial, but not in the BFD one (**Table 1**; **Figure 3**), likely because of the higher competitive efforts of hungry nestlings.

Table 1. Models of begging intensity (multinomial mixed models) and of probability of being fed after an own parent-absent begging (PAB) event (binomial mixed models), in before (BFD) or after (AFD) food deprivation trials (see Results). 'PAB category' is a three-level factor indicating whether begging intensity was recorded in feeding events occurring after an own PAB, a sibling's PAB, or when no PAB had occurred (see Methods).

	BFD trial			AFD trial		
	F/Z	*df*	*P*	*F/Z*	*df*	*P*
Begging intensity						
Experimental group	0.28	1, 22	0.60	17.64	1, 22	< 0.001
PAB category	5.35	2, 22	0.013	6.09	2, 22	0.008
Experimental group × PAB category	1.33	2, 22	0.29	6.02	2, 22	0.008
Probability of obtaining food						
Experimental group	0.36	-	0.74	2.25	-	0.025

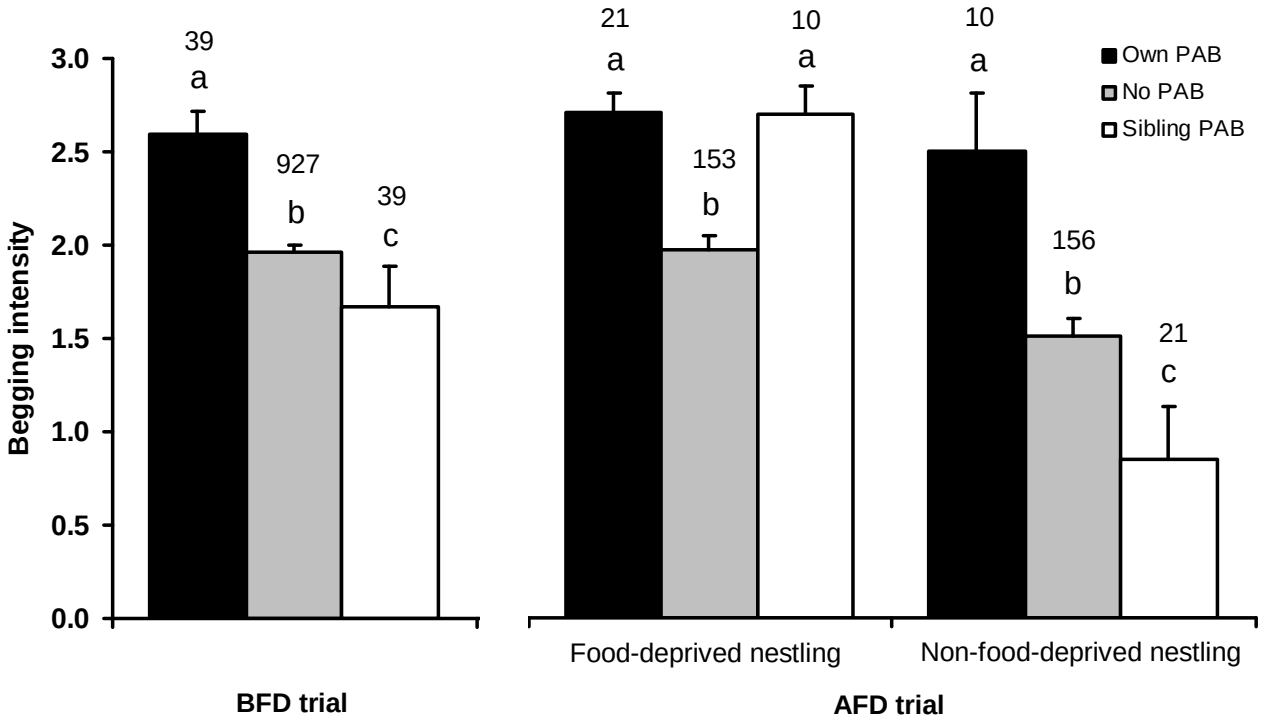

Figure 2. Mean (+ SE) begging intensity in feeding events occurring after an own ('own PAB') or sibling ('sibling's PAB') event of parent-absent begging (PAB), or when no PAB had occurred ('no PAB'). Mean values are reported separately for before (left panel, BFD) or after food deprivation (right panel, AFD) trials (see **Table 1** for statistical analyses). Values for AFD trials are shown for food-deprived and non-food-deprived nestlings separately. Numbers above bars denote the number of feeding visits for which we scored begging intensity. Letters indicate statistically significant differences ($P < 0.05$) as obtained by planned comparison tests and refer to comparisons among PAB categories (BFD trial), or among PAB categories for food-deprived and non-food deprived nestlings separately (AFD trial).

Interestingly, after an own PAB, in the AFD trial the non-food-deprived nestling was not more likely to obtain food than its nestmate (**Figure 3**).

DISCUSSION

We found that barn swallow nestlings increased the frequency of PAB displays when food-deprived. Moreover, during parental feeding events, nestlings that had performed PAB begged more intensely relative to their baseline begging levels, whereas their siblings reduced their own begging intensity. Finally, the chances of being fed were larger when a nestling had performed PAB before parental arrival. This pattern was more clear in the BFD trial, when nestlings were tested under the same hunger level, while AFD the response of nestlings to sibling's PAB, as well as the probability of obtaining food, varied according to the experimental group, as the food-deprived nestlings did not reduce begging intensity at parental arrival after a PAB by its non-food-deprived nestmate.

We interpret these findings as evidence that PAB reflects contingent need of food, similarly to parent-present begging (e.g. Boncoraglio et al., 2009; Romano et al., 2012), and that hungry nestlings are conveying to their nestmates in the absence of parents reliable information about their willingness to intensely beg for food at the following food provisioning

event, as demonstrated by their increased competitive effort after performing PAB. As a result, less needy nestlings toned down their begging levels at parental arrival, thus allowing for easier access to food by their siblings. It could be argued that reduction in the intensity of begging performed by a nestling upon a parental visit following PAB by its nestmate is caused by larger concomitant begging intensity by the nestling that performed PAB rather than by a response to PAB itself. This interpretation is unlikely, however, because the probability that a nestling did not beg significantly increased after a PAB by its sibling, suggesting that the observed reduction of begging intensity after a sibling's PAB mainly reflects a response to behavioural displays during inter-feeding intervals. Furthermore, we emphasize that, in the AFD trial, the non-food-deprived nestling maintained a high level of begging intensity during feeding visits preceded by its own PAB, as occurs in the BFD trial (**Figure 2**). This was the case despite the fact that its nestmate was food-deprived and thus much more needy, and indicates that the begging intensity after own PAB does not depend on the competitive environment experienced by the nestling, thereby corroborating the idea that PAB is a reliable signal of the willingness to intensely compete for the next delivered food item.

Such a fine-tuned modulation of begging displays by both the interacting siblings upon parental feeding visits following PAB suggests that in our model species PAB may be used by nestlings as a reliable information to decide whether escalating begging intensity during the next feeding events to outcompete nestmates or refraining from competition against a more vigorous competitor (Roulin et al., 2000; Johnstone and Roulin, 2003).

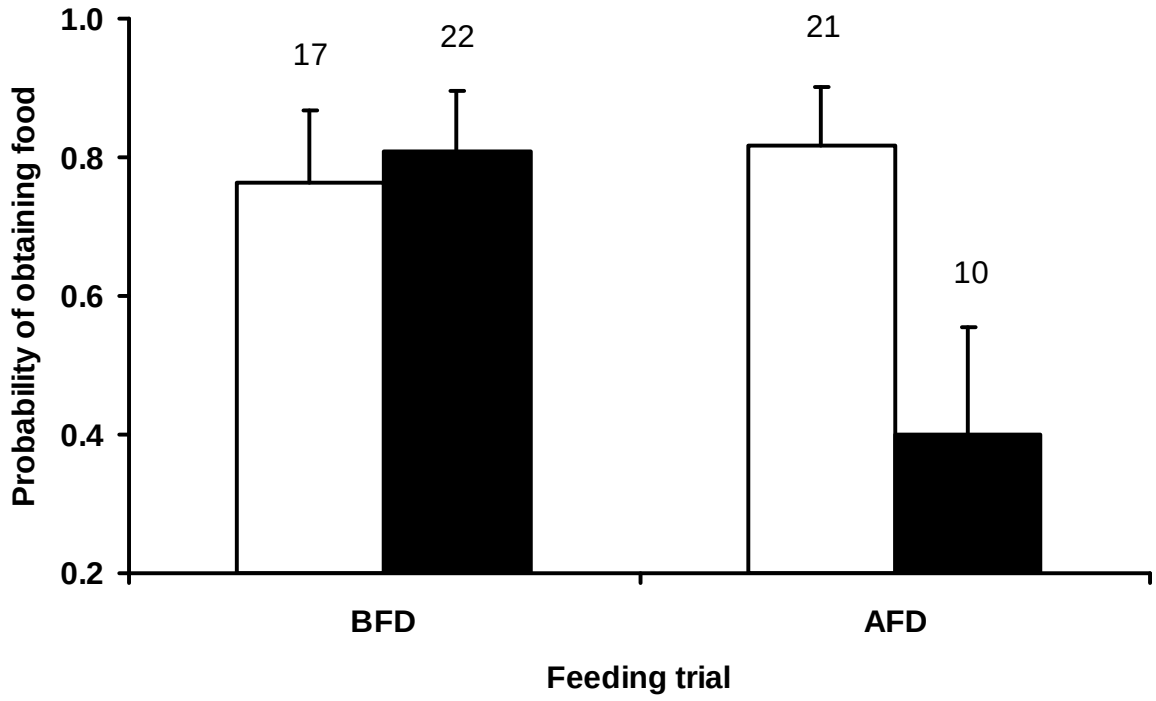

Figure 3. Probability (+ binomial SE) of obtaining the first feeding after an own event of parent-absent begging (PAB) for food-deprived (white bars) and non-food-deprived (black bars) before (BFD) and after (AFD) a period of food deprivation (see **Table 1** for statistical analyses). Note that if PAB has no effect on the probability of obtaining food at the subsequent feeding visit, the expected probability that each nestling obtains food after performing PAB is 0.5.

When exposed to a nestmate's PAB, a nestling may thus give up from escalating competition, because the indirect fitness benefits of securing access to food to a sibling in poor condition (see also Godfray, 1991) and the direct fitness advantage of saving energy for begging displays can outweigh the cost of missing a feeding event (Roulin et al., 2000; Johnstone and Roulin, 2003).

Proximately, PAB displays may be a consequence of hunger-induced low sensitivity in correctly detecting parental feeding visits (*sensu* Leonard and Horn, 2001; Leonard et al., 2005), which may serve to minimize the chances of missing food provisioning (Dor et al., 2007), especially when the marginal benefit of acquiring an additional unit of food is high (e.g. after food deprivation). Indeed, previous studies of passerines showed that PAB is generally elicited by several stimuli (see e.g. Leonard and Horn, 2001) and is apparently not directed towards nestmates. Nevertheless, our findings suggest that natural selection may have promoted the ability of nestlings at reciprocally exploiting such 'false alarms' to identify highly competitive siblings in order to modulate their begging behaviour accordingly and reduce intensity of scramble competition. Therefore, PAB displays in barn swallow nestlings may have the dual adaptive function of minimizing the chances of missing feedings (Dor et al., 2007) and reducing the costs of scrambling when facing hungry, highly competitive siblings, that are more likely to obtain the subsequent parental food item.

Besides testing if PAB is used by siblings to reciprocally adjust competitive efforts under a normal feeding regime, we also evaluated whether its expression was context-dependent, i.e. whether experimentally food-deprived nestlings behaved differently in response to own or sibling's PAB compared to non-food-deprived, normally fed, nestmates. The results were neat in showing that food-deprived nestlings did not respond to a sibling's PAB by reducing their begging intensity, as they conversely did when non-food-deprived, but rather escalated competitive efforts by begging at a significantly higher intensity than their baseline begging level (**Figure 2**). Thus, PAB by non-food-deprived nestmates exacerbated competition efforts by food-deprived siblings when parents arrived at the nest with food, again indicating that PAB, irrespective of mechanisms that generates it, induces a strong behavioural response by the interacting siblings. Such competitive escalation affected access to food in the AFD trial, given that, after an own PAB, non-food-deprived nestlings were not more likely to obtain food than their food-deprived nestmates not performing PAB (see **Figure 3**). This novel finding indicates that marked competitive asymmetries in need between otherwise similar siblings strongly affect the behavioral response of the needier nestlings to PAB uttered by their less needy nestmates. Food-deprived nestlings responded to their nestmates' PAB displays by vigorously competing with them to obtain the next food item, rather than reducing their competition efforts as in the BFD trial, resulting in equal chances of getting food as their less needy siblings. Under such circumstances, it is likely that the benefit of obtaining an additional food item was disproportionately larger for the food-deprived than for the non-food-deprived nestling. Indeed, for the food-deprived nestling the potential cost of refraining from competition could be too large, and probably not compensated by the indirect fitness benefit

of favouring the access to food by a less needy sibling. Hence, whenever large asymmetries in hunger level between otherwise similar siblings exist, PAB may have the counterproductive effects of stimulating competition by the more needy nestmate, suggesting that PAB in the study species, despite being perceived and used by nestmates to modulate their behaviour, constitutes a sort of 'false alarm'. We can also safely rule out the possibility that the escalating competitive behaviour of food-deprived nestlings after non-food deprived nestmates PAB was caused by a low-intensity PAB signal by non-food-deprived siblings, that may not convey sufficient information about willingness to compete for the subsequent food item: we did not find any statistically significant difference in intensity of PAB signals between food- and non-food-deprived nestlings (details not shown for brevity).

Previous studies investigating the evolutionary functions of PAB mostly focused on barn owl (*Tyto alba*) nestlings, that 'negotiate' priority in scrambling for food before arrival of parents at the nest via a large and complex repertoire of within-brood communication signals almost before any feeding visit (Roulin et al., 2000; Roulin, 2001; Roulin, 2004; Roulin et al., 2009; Dreiss et al., 2010). Such an intense negotiation among nestlings, resulting in a reciprocal modulation of competitive behaviour for access to food in response to own or nestmates' PAB signals (Dreiss et al., 2010), has the two-fold advantage of reducing the costs of competition and increasing their indirect fitness (Roulin et al., 2000; Johnstone and Roulin, 2003). On the other hand, most studies of PAB in other bird species did not investigate whether PAB is involved in intra-brood communication (Budden and Wright, 2001; Leonard and Horn, 2001; Rivers et al., 2001; Leonard et al., 2005; Dor et al., 2007). A single study demonstrated modulation of PAB behaviour in response to experimentally broadcasted PAB signals, but did not analyse the variation of begging behaviour at parental arrival nor the consequences in terms of food intake (Bulmer et al., 2008). Remarkably, in the barn swallow the effect on the outcome of sibling competition following a PAB, despite not being preceded by prolonged and reciprocal 'negotiation' displays among nestlings as occurs in barn owl broods, is compatible with that observed in the latter species. Our findings thus corroborate the idea that offspring may significantly impact on feeding decisions by parents via intra-brood signalling dynamics.

In conclusion, we suggest that nestling barn swallows, by exploiting PAB signals by siblings, may strive to achieve an optimal balance between the overall costs of sibling competition, the direct fitness benefits of obtaining food by parents and the indirect fitness costs of subtracting food to needy kin, while additionally reducing the chances of missing feeding events. However, we also experimentally demonstrated that strong asymmetries in individual need may lead competing siblings, especially under food shortage, to attempt to monopolize parental resources.

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Chapter 3

Sex-related effects of an immune challenge on growth and begging behavior of barn swallow nestlings

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Sex-Related Effects of an Immune Challenge on Growth and Begging Behavior of Barn Swallow Nestlings

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Abstract

Parent-offspring conflicts lead the offspring to evolve reliable signals of individual quality, including parasite burden, which may allow parents to adaptively modulate investment in the progeny. Sex-related variation in offspring reproductive value, however, may entail differential investment in sons and daughters. Here, we experimentally manipulated offspring condition in the barn swallow (*Hirundo rustica*) by subjecting nestlings to an immune challenge (injection with bacterial lipopolysaccharide, LPS) that simulates a bacterial infection, and assessed the effects on growth, feather quality, expression of morphological (gape coloration) and behavioral (posture) begging displays involved in parent-offspring communication, as well as on food allocation by parents. Compared to sham-injected controls, LPS-treated chicks suffered a depression of body mass and a reduction of palate color saturation. In addition, LPS treatment resulted in lower feather quality, with an increase in the occurrence of fault bars on wing feathers. The color of beak flanges, feather growth and the intensity of postural begging were affected by LPS treatment only in females, suggesting that chicks of either sex are differently susceptible to the immune challenge. However, irrespective of the effects of LPS, parents equally allocated food among control and challenged offspring both under normal food provisioning and after a short period of food deprivation of the chicks. These results indicate that bacterial infection and the associated immune response entail different costs to offspring of either sex, but a decrease in nestling conditions does not affect parental care allocation, possibly because the barn swallow adopts a brood-survival strategy. Finally, we showed that physiological stress induced by pathogens impairs plumage quality, a previously neglected major negative impact of bacterial infection which could severely affect fitness, particularly among long-distance migratory birds.

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Introduction

Theoretical models of conflicts among family members posit that offspring are selected to obtain a larger share of parental resources than their siblings and to attract more care than would be optimal for parents to provide [1–3]. Because an even investment in offspring with different quality may result in a waste of reproductive effort, parents may decide to differentially invest limiting resources in relation to the reproductive value of individual offspring, in order to maximize their own fitness [2–6].

In altricial species from diverse taxa, offspring are entirely dependent on food provided by parents and solicit care by using morphological and behavioral 'begging' displays [7,8]. Natural selection may thus have promoted the parental ability to allocate resources according to variation in offspring signals of need (e.g. hunger) and condition (general state) (see [9]). Indeed, both theoretical models and experimental studies have supported this prediction, and have suggested that multi-trait begging displays convey reliable information over offspring quality to attending parents [2,3,7,10–13].

Given the abundance of parasites in natural environments, one important source of variation in offspring quality is parasite infection [14–16]. Parasites can negatively influence the physiological state of their hosts by causing disease and reducing food intake and resource assimilation [17,18], or imposing an energy cost due to mounting an immune response which may have to be traded against competing physiological functions [19–21]. Negative effects of parasite infection should be more intense in young individuals that have a relatively naïve immune system [14,16], reducing growth and survival [17,19]. In birds, different components of offspring begging, such as postural and vocal displays and gape coloration, may reveal infection by parasites, thus potentially allowing parents to invest resources differentially according to progeny current level of infection [13,15,22,23]. Begging displays may thus function as 'honest' signals of offspring general condition and/or reveal need of food [2,3,7,10,11,13,15], and several studies have demonstrated that parents respond to them by increasing food provisioning [7,9,24–27]. Importantly, the marginal fitness return of investing in offspring of different condition may vary according to contingent need of food by individual nestlings [15,25,27,28].

Another crucial source of variation in offspring reproductive value is sex, as males and females may differ in susceptibility to environmental and rearing conditions [27,29] as well as in their

food demands, competitive ability and begging behavior [25,30]. Sex-related variation in offspring fitness returns may thus promote differential parental investment in sons and daughters [4,6]. However, despite the important role of sex in determining developmental trajectories, physiology and behavior [31], sex-specific susceptibility to parasitism of the offspring has been seldom investigated in avian species [32–34].

The aim of this study of barn swallow (*Hirundo rustica*) nestlings was to evaluate whether an immune challenge, simulating an infection by a bacterial pathogen, affected body mass and feather growth, as well as the expression of morphological (gape coloration) and behavioral (postural display) begging traits involved in parent-offspring communication. In addition, we evaluated variation in parental allocation strategies towards offspring differing in condition, as affected by the immune challenge, as well as by contingent need of food, as experimentally altered by a short-term food deprivation, because parental decisions and begging behavior are also expected to vary in relation to both general condition and current hunger state of the offspring (see [9]). Finally, we investigated whether male and female chicks responded differently to the immune challenge.

We simulated a bacterial infection by injecting half of the chicks of a brood with lipopolysaccharide (LPS) (LPS chicks), an endotoxin extracted from the outer membrane of Gram-negative bacteria, and the other half with a saline control solution (control chicks). LPS is commonly used to elicit an immune response in the absence of a living pathogen and causes several hormonal and behavioral alterations (the 'sickness behavior syndrome') in birds (e.g. [17,18]).

We predicted that LPS depressed body mass and resulted in lower plumage quality [17,18,35], as gauged by feather growth and occurrence of fault bars. Fault bars are translucent bands on feathers, running perpendicular to the rachis, caused by defective development of barbules [36]. We also predicted that exposure to LPS resulted in paler coloration of chick gapes [22]. However, we had no specific predictions concerning the differential effects of LPS treatment on male and female offspring.

Finally, two days after injection, we experimentally tested the effects of the immune challenge on success in sib-sib competition between LPS and control nestlings. We measured the intensity of postural begging behavior, reflecting the degree of offspring need, and the change in body mass during feeding trials. Moreover, we counted the number of feedings provided by parents to each chick, in tests where pairs ('dyads') of same-sex and opposite-treatment brood-mates were set to compete for parental feedings [25,26,28].

Materials and Methods

General field procedures and sex determination

The barn swallow is a small (ca. 20 g), insectivorous migratory passerine with biparental care of the offspring. Females lay 1–3 clutches of 1–7 eggs (modal size: 5 eggs) per breeding season [37]. Nestlings hatch approximately 14 days after the onset of incubation, and fledge when they are 19–21 days old [37].

The present study was carried out between April and July 2010 at two colonies (n = 58 breeding pairs in total) located near Milan (Northern Italy). Starting from April 1st, nests were visited daily to record breeding events. At day 7 (day 0 = hatching of the first egg in a nest) we ringed all the chicks from broods with three or more nestlings and collected a blood sample (ca. 80 µl) for molecular sexing by PCR amplification of the sex-specific avian *CHD-1* gene following the protocol originally devised by Griffiths et al. [38], slightly modified according to Saino et al. [39]. This procedure allowed us to determine the sex of all nestlings before the day of the immune challenge.

On day 12 (mean ± SD: 11.93±0.62 days), when chicks have attained final body size and before the onset of pre-fledging mass recession [40], we intraperitoneally injected half of the male and half of the female chicks within each brood (107 males and 95 females from 47 broods) with 20 µl phosphate-buffered saline (PBS) containing 10 µg of lyophilized LPS powder, isolated from *Escherichia coli* (055:B5 - L2880 Sigma-Aldrich) (e.g. [17,41]). Injection with LPS provokes a rapidly ensuing innate immune response ('acute phase response'). The acute phase response triggers neuroendocrine processes, such as inhibition of the hypothalamo-pituitary-gonadal axis and activation of the hypothalamo-pituitary-adrenal axis, by release of glucocorticoids [18]. In passerines, in conjunction with these hormonal alterations, LPS causes a typical 'sickness behavior' by reducing activity and food intake, inducing somnolence and hypothermia, and often resulting in mass loss [17,18,41,42]. Since body mass of barn swallow chicks at day 12 is ca. 20 g (20.66 g±0.12 SE in our sample of nestlings), the amount of LPS we chose to inject corresponds to ca. 0.5 µg g^{-1} body mass, a dose similar to that used in previous studies of passerines (e.g. [21,43,44]). The remaining nestlings were injected with the same amount of phosphate-buffered saline (PBS) to serve as controls. For example, in a brood containing two male and two female nestlings, we injected one male and one female with LPS, and one male and one female with PBS. Nestlings were assigned to the LPS or control group randomly. In case of an odd number of nestlings of either sex, the odd nestling was assigned randomly to either treatment. Overall, we injected 102 nestlings with LPS (56 males and 46 females) and 100 with PBS (51 males and 49 females).

Before LPS injection, we measured body mass to the nearest 0.1 g by an electronic balance and tarsus length with a digital caliper (to the nearest 0.01 mm). The length of all primary wing feathers (hereafter 'primary feathers') was also measured using a ruler (to the nearest 0.5 mm). As a proxy of wing length, we used the length of the third outermost right primary feather ('feather length' hereafter) [45]. Body mass, tarsus length and feather length were measured again 2 and 3 days after the injection (i.e. at day 14 and 15) to evaluate the effects of LPS on growth.

Finally, on day 17 we counted the fault bars on both wings in a subsample of 75 LPS and 73 control chicks from 35 broods. To determine which fault bars were developed after LPS treatment, we measured the length of all the feathers where fault bars were found as well the distance between each fault bar and the tip of these feathers. Since we already knew the length of all feathers before the injection of LPS or PBS, this procedure allowed us to determine the number of fault bars appearing after the immune challenge.

Mouth coloration

On day 12 (before LPS injection), we recorded mouth coloration of 149 chicks (75 LPS and 74 control) belonging to 35 broods with a spectrometer powered by a deuterium-tungsten halogen light source (Avantes AvaSpec 2048). The reflectance (%) of the mouth was measured relative to a standard white tablet (WS-2). To prevent interference by stray light, the reflection probe was positioned inside a matte black plastic tube, cut at 45° in order to avoid specular reflection when the probe was applied to the mouth. The illuminated field was about 7 mm^2 and every reading was obtained from an average of 15 scans. Each nestling was measured twice in two regions of the gape, corresponding to the left flange and the palate. The standard white was recalibrated before starting measurements of any next brood and the white

periodically checked to verify 100% reflectance. Reflectance measures were repeated 2 and 3 days after the injection of LPS.

Color analyses were restricted to the 320–700 nm spectral window, corresponding to the typical visual range of passerines (see [46]). Spectral color composition was summarized by computing brightness, chroma and hue according to the segment classification method developed by Endler [47] and using the formulas given by Armenta et al. [46], employing *ad hoc* implemented macros for Microsoft Office Excel 2003. Brightness corresponds to the total reflectance of a given surface, chroma represents the spectral purity (saturation), while hue is the spectral location, representing the position of a spectrum in the color wheel, progressing from red to UV-A. Repeatability of the two reflectance measures of each gape region was high, with intraclass correlation coefficient [48] ranging between 0.613 ($F_{1,147} = 4.152$; $P<0.0001$) and 0.893 ($F_{1,148} = 17.728$; $P<0.0001$). Brightness, chroma and hue were thus averaged between spectra before analyses.

Feeding trials and video recordings of begging

To test for a difference in competitive ability between LPS and control chicks, we compared the intensity of postural begging, body mass gain and number of feedings received from parents during feeding trials within pairs of same-sex and opposite-treatment siblings (i.e. either LPS male vs. control male or LPS female vs. control female) two days after experimental treatment. Pairs of same-sex chicks (dyads hereafter) were randomly chosen within each brood. The test was performed both before and after a period of food deprivation in order to analyze the behavior of nestlings and parents under different hunger conditions (normal food intake vs. hunger condition) [25,26]. The main aim of feeding trials was to evaluate the effect of the immune challenge on competitive interactions between LPS and control nestlings. Because male and female barn swallow nestlings differ in competitive ability [25,49] and are differently susceptible to poor rearing condition [27], including parasite loads [33], in order to experimentally control for the effect of sex we decided to establish only dyads of nestlings of the same sex.

First, the two focal nestlings were weighed, individually marked on their head with two white spots, and left in the nest for a feeding trial while temporarily removing the other chicks, that were kept in a safe and warm place. All feeding visits of the parents were videotaped with a Sony DCR-SR72E camera, placed 2–3 m from the nest in a frontal position. Recordings started in the morning at 7.30 A.M. ($\pm$30 min). At the end of the 1.5 h feeding trial, the focal nestlings were weighed again in order to record body mass gain, indicating individual food intake [25]. Afterwards, they were placed in a warm cloth bag and in a safe position for 2 h of food deprivation while their siblings were put back in the nest. Food deprivation was intended to simulate a short period of starvation, similar to what may naturally occur, for example, in case of heavy rain. The same procedure was repeated in a second feeding trial, performed after the 2 h of food deprivation. Finally, all nestlings of the brood were returned to the nest.

The number of feedings obtained by each nestling of the dyad were counted on video recordings using VLC Media Player 1.1.4 (Free Software Foundation, Inc., Boston, MA). The use of the number of feedings provided by parents to each chick during trials was intended to assess the ability of the nestlings in sib-sib competition as number of interactions won against their competitors. Moreover, because feeding rates do not account for variation in size of individual feedings, we also used body mass change during each trial as a proxy for food intake and the balance between benefits and costs of scrambling.

Furthermore, three feeding events were randomly chosen to estimate the intensity of postural begging, which was scored on a four-levels scale ranging between 0 (the nestling did not beg) and 3 (the nestling begged by moving the open wide mouth with fully stretched neck and tarsi) [26]. Feeding events were selected over the entire duration of recordings (1.5 h) for the first feeding trial while only over the first half (45 min) for the trial performed after food deprivation in order to avoid the dissipation of any effect of increased hunger level on the intensity of postural begging (see [26]). Begging scores were then averaged for each chick within a trial. All these measures were taken blindly with respect to treatment. The analyses of postural begging displays were performed both by using average begging scores for each chick within a trial as well as all the three measures for each chick within a trial.

The whole protocol was performed for 45 dyads (24 male and 21 female dyads) belonging to 39 different broods.

Statistical analyses

The effects of immune challenge on nestling traits (body mass, feather length, color hue, chroma and brightness of both flange and palate) were analysed using linear mixed models, which included as predictors two dichotomous fixed factors for treatment (LPS or control) and sex, their interaction, and the value of the trait of interest before LPS injection as a covariate. Nest identity was included in the models as a random intercept effect. In all analyses, we also included age at LPS injection as a covariate to account for small variation in age at measurement. We first run separate analyses for trait values recorded 2 and 3 days after LPS injection, and then analysed traits recorded in both days in the same model, while including an additional fixed factor (day of measurement), identifying the data collected in either day.

The presence and number of fault bars on the wings after LPS injection were analysed in mixed models assuming a binomial and a Poisson error distribution, respectively. The presence and number of fault bars before LPS injection were also included as predictors in the relevant models to account for individual variation in the number of fault bars at the beginning of the test. Nest identity was included as a random factor in the models.

The analyses of postural begging intensity, number of feedings and body mass gain during feeding trials were carried out using repeated-measures linear mixed models where nestlings were modeled as subjects. Food deprivation (before or after) was included as a dichotomous factor identifying the repeated measurements of each subject. Sex, treatment (LPS or control) and the interaction between treatment and food deprivation were included as fixed factors. Nest and dyad were included as random intercepts, and the effect of food deprivation was allowed to vary randomly between chicks (random slope model) [50]. Because begging intensity and the outcome of sibling competition may depend both on the sex of the focal nestling and on the sex of the competitor [49], and dyads always included chicks of the same sex (see above), our experimental design did not allow to analyze the effect of the statistical interaction between sex and treatment. We therefore ran the analyses for each sex separately.

All analyses were run in R 2.8.1 (R Development Core Team 2008) with the *lmer* procedure of the *lme4* package [51]. P-values for linear mixed models were calculated by means of the likelihood ratio statistic [52]. Interaction terms were removed from the models if not significant ($P>0.05$). Sample size may differ slightly between different analyses because of missing data for some chicks.

Ethics statement

When removed from their nest, nestlings were kept in a safe and warm place. At each measurement session each chick was handled

only for few minutes and nests were never left without at least one nestling inside to avoid parental desertion. Blood samples were collected by slightly puncturing the brachial vein and the puncturing site was carefully disinfected. Injections of LPS and PBS were performed just below the pectoral muscle threading the tiny needle (30 G) approximately 3–4 mm inside the abdominal cavity and taking care to avoid damaging inner organs (which are easily visible through the skin at this age). No obvious negative consequences of handling nestlings were detected. Nestling mortality until fledging was very low (2 out of 102 LPS chicks and 2 out of 100 control nestlings), and unaffected by experimental treatment. During videotaping, we could not note any obvious effect derived from the presence of recording equipment on both parental and offspring behavior. The study was approved by the Ethical Committee of the Department of Biology, University of Milan, Italy.

Results

Body mass, feather growth and occurrence of fault bars

Before LPS treatment, no significant differences in body mass, tarsus or feather length were found between LPS and control nestlings (in all cases: $\chi^2_1 \leq 2.339$; $P \geq 0.126$).

On both day 2 and 3 after injection, body mass of LPS nestlings was significantly lower than that of their control siblings (Table 1; Figure 1), whereas it did not differ between sexes. On day 2 post-injection, feather length was differently affected by LPS depending on sex, with LPS females, but not males, growing shorter feathers than controls (Table 1; Figure 2), though this effect was no longer significant on day 3 post-injection (Table 1; Figure 2).

Analyses run by including data from both day 2 and 3 post-injection in the same model confirmed the statistically significant effect of immune challenge on body mass ($\chi^2_1 = 15.460$; $P < 0.0001$), while the effect of treatment on feather length was non-significant ($\chi^2_1 = 2.603$; $P = 0.107$). All the two- and three-way interaction terms between day of measurement, sex and treatment were not statistically significant (in all cases: $\chi^2_1 \leq 2.792$; $P \geq 0.095$), implying that the effects of immune challenge did not differ statistically between day 2 and 3 post-injection.

Table 1. Effect of LPS on body mass and feather length on day 2 and 3 post-injection.

Source of variation	Coefficient	χ^2	df	P
Day 2 post-injection				
Body mass (n = 202)				
Treatment	−0.492 (0.087)	29.25	1	<0.0001
Sex	0.089 (0.103)	0.74	1	0.388
Feather length (n = 201)				
Treatment	−0.684 (0.321)	4.59	1	0.032
Sex	−0.647 (0.342)	1.90	1	0.168
Treatment × sex	−1.090 (0.444)	6.07	1	0.014
Day 3 post-injection				
Body mass (n = 193)				
Treatment	−0.251 (0.114)	4.83	1	0.028
Sex	0.053 (0.133)	0.16	1	0.691
Feather length (n = 193)				
Treatment	−0.244 (0.247)	0.99	1	0.320
Sex	0.216 (0.278)	0.62	1	0.430

Final models reporting the effect of treatment (LPS or control), sex and their interaction (where significant) on body mass and third primary feather length of nestlings both two and three days after the experimental manipulation. The number of nestlings in the sample is given in parentheses. See Statistical Methods for details.

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Before LPS injection, no differences in the presence and in the number of fault bars on wing feathers were found between LPS and control nestlings (in both cases: $|z| \leq 0.860$; $P \geq 0.388$). Conversely, post-injection, both the proportion of individuals with fault bars on wing feathers and the number of fault bars differed between treatments (Figure 3): fault bars were found on 26 out of 75 LPS nestlings but only on 12 out of 73 control chicks ($z = 2.384$; $P = 0.017$), while the absolute number of fault bars present on LPS nestlings was more than twice that on controls (Figure 3; $z = 2.808$; $P = 0.005$). No differences in presence and number of fault bars were found between male and female nestlings (in all cases: $|z| \leq 1.401$; $P \geq 0.161$). The treatment by sex interaction was not significant (in all cases: $|z| \leq 0.170$; $P \geq 0.865$) and was therefore removed from the models.

Mouth coloration

Before LPS treatment, no significant differences in brightness, chroma and hue of the palate and the flanges were found between LPS and control nestlings ($\chi^2_1 \leq 2.456$; $P \geq 0.117$ for all color variables).

On day 2 after treatment, chroma was affected by LPS. In the palate, chroma was significantly smaller in LPS than in control nestlings (control: 0.251±0.008; LPS: 0.236±0.008; $\chi^2_1 = 6.278$; $P = 0.012$; Table S1), while flange chroma was significantly affected by the interaction between sex and treatment ($\chi^2_1 = 6.026$; $P = 0.014$; Table S1), with LPS reducing chroma of females (control: 0.095±0.005 SE; LPS: 0.079±0.005 SE) but not males. A sex-related variation also emerged within the control group with males having larger flange chroma than females ($\chi^2_1 = 7.950$; $P = 0.005$; Table S1). Finally, females had larger flange hue than males ($\chi^2_1 = 6.301$; $P = 0.012$; Table S1), while LPS and control nestlings did not differ for this variable ($\chi^2_1 = 2.169$; $P = 0.141$).

On day 3 post-injection, no effects of treatment (in all cases: $\chi^2_1 \leq 2.231$; $P \geq 0.135$) or sex (in all cases: $\chi^2_1 \leq 3.470$; $P \geq 0.062$) were found on any color variable (Table 2).

Analyses including data from both day 2 and 3 after LPS injection (see Statistical analyses) confirmed the significant main effect of the immune challenge on palate chroma ($\chi^2_1 = 4.951$; $P = 0.026$) as well as of the interaction between sex and LPS treatment on flange chroma ($\chi^2_1 = 4.556$; $P = 0.033$). These models also confirmed the sex-related variation in the flange chroma ($\chi^2_1 = 4.746$; $P = 0.029$) (see above). In addition, the other two- and three-way interaction terms between day of measurement, sex and/or LPS were not statistically significant (in all cases: $\chi^2_1 \leq 3.116$; $P \geq 0.078$), implying that the effects of LPS treatment were not significantly different between day 2 and 3.

Feeding trials

Repeated-measures mixed models showed that average begging intensity was higher among LPS compared to control chicks (Table 2), whereas there was no effect of treatment on food allocation and body mass gain during feeding trials (Table 2). Average begging intensity, the number of feedings received per capita and body mass gain during feeding trials significantly increased after food deprivation (Table 2).

Separate analyses for each sex revealed no significant effect of LPS treatment on average begging intensity, food allocation and body mass gain in males, though all these variables were strongly

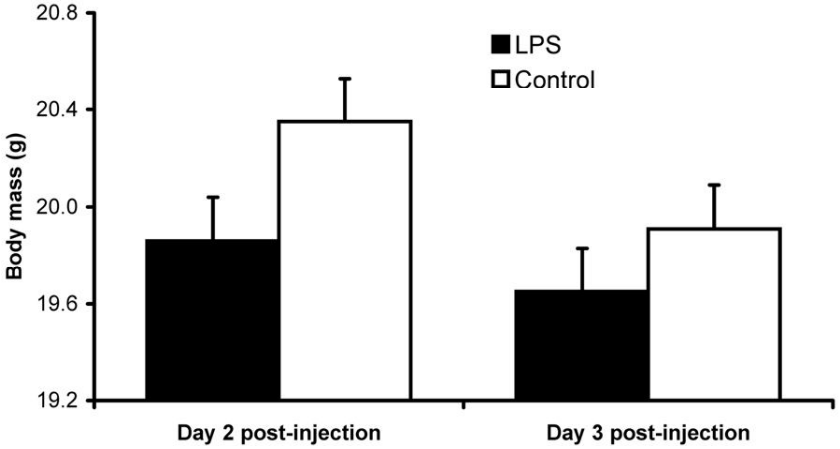

Figure 1. Body mass on day 2 and 3 after LPS injection. Model-estimated (see Table 1) mean body mass (+ SE) of LPS and control nestlings 2 (left) or 3 (right) days after the immune challenge.
doi:10.1371/journal.pone.0022805.g001

affected by food deprivation (Figure 4; Table 2). Conversely, LPS females begged more intensely than their control sisters (Figure 4) but did not receive more food from parents nor did they gain more mass (Table 2). Interestingly, among female nestlings, both the number of feedings obtained and the body mass gain significantly increased after food deprivation, while begging intensity increased slightly but not significantly (Table 2).

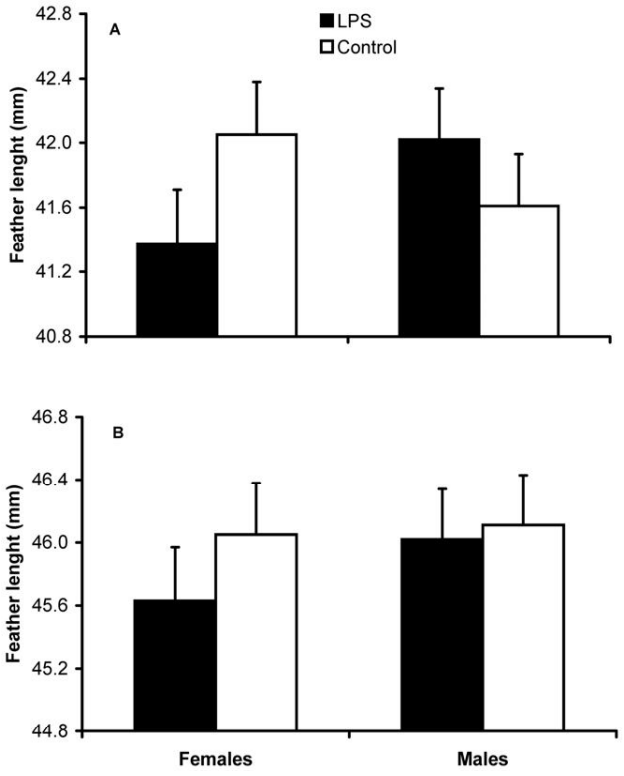

Figure 2. Feather length of males and females on day 2 and 3 after LPS injection. Model-estimated (see Table 1) mean third primary feather length of male and female nestlings belonging to the LPS or control group 2 (A) and 3 (B) days after the immune challenge.
doi:10.1371/journal.pone.0022805.g002

Qualitatively similar results were obtained by including in the model all three available scores of begging intensity for each chick rather than using individual averages computed within trial (details not shown for brevity).

Discussion

In this experiment on barn swallow nestlings, we subjected chicks to an immune challenge with LPS, and assessed its effects on growth, feather quality and parent-offspring communication. Infection by Gram-negative bacteria can elicit an immune response and cause an 'acute phase response', entailing physiological and behavioral alterations [18,42]. As predicted, the immune challenge negatively affected several morphological traits such as body mass, palate color and feather quality as reflected by the occurrence of fault bars on wing feathers. The effect of LPS on wing feathers growth and beak flange coloration differed between males and females, as suggested by the significant sex by treatment interaction. Moreover, the LPS injection determined an increase in begging intensity of females but not of males. Albeit a direct comparison between sexes was prevented by our experimental design (see Methods), the difference in begging intensity between

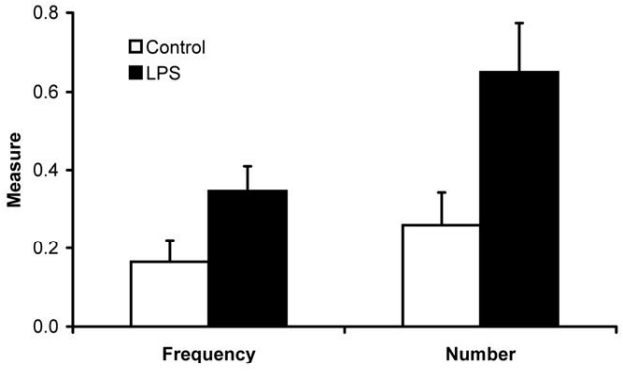

Figure 3. Frequency and number of fault bars on feathers in relation to LPS treatment. Proportion (+ SE) of individuals with fault bars (left) and mean number of fault bars (right) on the wings feathers of LPS (n = 75) and control (n = 73) chicks. Standard errors were calculated using the Wilson's score method incorporating continuity correction.
doi:10.1371/journal.pone.0022805.g003

Table 2. Effect of LPS on intensity of postural begging, number of feedings received and body mass gain during feeding trials.

Source of variation	Coefficient	$\chi 2$	df	P
All nestlings				
Postural begging (n = 90)				
Sex	−0.092 (0.153)	0.362	1	0.548
Treatment	0.236 (0.107)	4.570	1	0.033
Food deprivation	0.184 (0.087)	4.436	1	0.035
Number of feedings (n = 90)				
Sex	−0.256 (1.332)	0.054	1	0.816
Treatment	0.050 (0.590)	0.007	1	0.935
Food deprivation	4.965 (0.633)	47.995	1	<0.0001
Body mass variation (n = 90)				
Sex	0.097 (0.119)	0.676	1	0.411
Treatment	0.008 (0.047)	0.029	1	0.865
Food deprivation	0.537 (0.064)	52.694	1	<0.0001
Male nestlings				
Postural begging (n = 48)				
Treatment	0.123 (0.142)	0.765	1	0.382
Food deprivation	0.286 (0.120)	5.367	1	0.021
Number of feedings (n = 48)				
Treatment	−0.376 (0.925)	0.172	1	0.678
Food deprivation	5.495 (0.977)	24.632	1	<0.0001
Body mass variation (n = 48)				
Treatment	0.008 (0.075)	0.011	1	0.915
Food deprivation	0.494 (0.090)	29.162	1	<0.0001
Female nestlings				
Postural begging (n = 42)				
Treatment	0.366 (0.161)	4.939	1	0.026
Food deprivation	0.082 (0.122)	0.466	1	0.495
Number of feedings (n = 42)				
Treatment	0.937 (0.619)	1.959	1	0.162
Food deprivation	4.334 (0.757)	29.168	1	<0.0001
Body mass variation (n = 42)				
Treatment	0.010 (0.053)	0.037	1	0.847
Food deprivation	0.059 (0.090)	29.733	1	<0.0001

Final models reporting the effect of treatment and food deprivation on postural begging, feedings received by individual offspring and body mass variation of nestlings in a repeated-measures linear mixed model. The analyses were performed for the entire set of nestlings, and for each sex separately. The number of nestlings in the sample is given in parentheses. See Statistical Methods for details.

doi:10.1371/journal.pone.0022805.t002

LPS and control females was three times larger than that observed among males, thus suggesting that also in this case the effect of the immune challenge may be sex-specific. However, LPS injection did not affect parental feeding effort both under a normal food provisioning regime and after a short period of food deprivation. Below we discuss the main findings.

Effects on morphological traits and feather quality

Loss of body mass and reduced feather development and quality (as gauged by the slower growth of primary feathers in females and by a larger occurrence of fault bars) following LPS challenge may have been caused by a smaller food intake. In passerine birds the acute phase response is associated with an increase in resting and somnolence as well as a reduction of behaviors associated with motility, like scrambling for food and sib-sib competitive interactions (the 'sickness behavior syndrome') [17,18,41,42]. These behavioral changes are typically short-lasting and individuals recover within 24 hours [18,42]. Nevertheless, other effects such as mass loss and growth reduction may persist for longer [17,18,41].

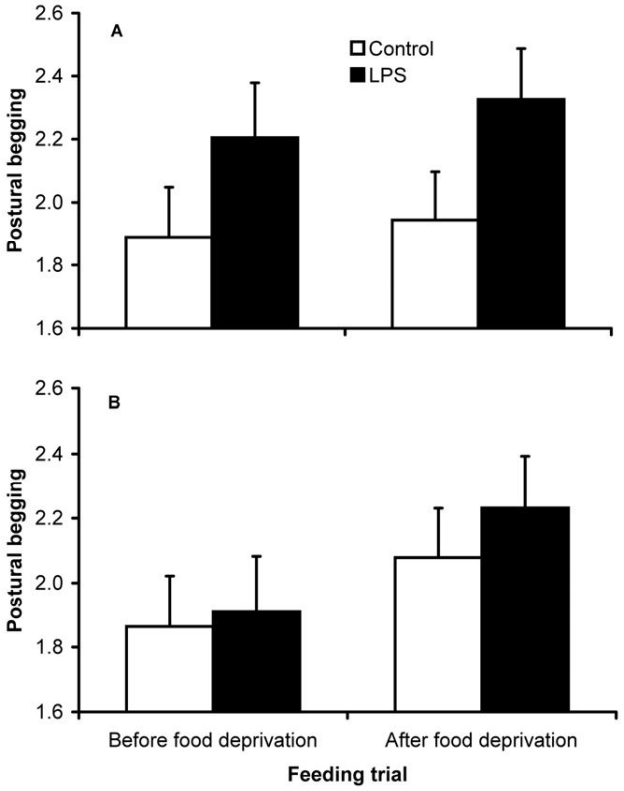

Figure 4. Begging intensity before and after food deprivation in relation to LPS treatment. Model-estimated (see Table 2) mean intensity (+ SE) of postural begging display in 21 dyads of female (A) and 24 dyads of male (B) nestlings, before and after a period of 2 hours of food deprivation.

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A decrease in body mass may also have been caused by a reduction in parental feeding soon after the immune challenge, either because LPS chicks may appear of reduced reproductive value to parents, or because their nest-mates prevailed in sib-sib interactions for access to food. Although we admittedly could not discriminate between these interpretations, we favor the idea that reduced access to food was mainly due to reduced motility. This is the case because previous studies on barn swallows suggested that both parents and older/larger chicks seem to enhance access to food by smaller nestlings, as expected in a species adopting a brood survival strategy [25,26,28,49].

Our findings also highlighted a possible trade-off between growth and immunity [53,54]. Functioning of the immune system is costly, and energy trade-offs among competing functions may be more intense in rapidly growing young individuals [55,56]. LPS nestlings may thus have used most of their available energy to mount an immune response, thus suffering a reduction in their body and feather growth, as observed in other bird species [19,20,35].

A novel finding is that the immune challenge increased the occurrence of fault bars, that are commonly considered as evidence of low feather quality [36]. Indeed, their presence is associated with higher risk of breakage [57,58] and with other major feather damages [59], that may result in a considerable reduction in aerodynamics and flight performance [60]. Feather damage may thus have consequences for aerial foraging, predator escape behavior, and migration performance [58] in this long-distance migratory bird. This finding corroborates previous evidence of impaired plumage growth consequent to an immune stimulation in molting adult house sparrows (*Passer domesticus*) [35]. Though the proximate mechanisms remain unknown, the negative effects of LPS injection on feather quality and growth may be mediated by corticosterone. Exposure to LPS is known to raise circulating corticosterone levels [18] and high corticosterone, in turn, reduces the number of barbules and affects their reciprocal distance [61,62], resulting in increased frequency of fault bars. Moreover, because nutritional stress is a main cause of fault bars [63,64], the effects of a corticosterone-mediated pathway may have been amplified by decreased food intake, as nutritional stress is known to significantly increase circulating corticosterone [63,64].

Effects on parent-offspring communication and sibling competition

We found that saturation of gape color, which is a main component of offspring begging signals, was depressed by LPS. The coloration of the soft tissues of the gape is partly dependent on the presence of dietary carotenoids [43,65], and a reduced chroma of the palate (both sexes) and flanges (females only) in LPS nestlings may therefore reflect a reduction in carotenoid assimilation. Furthermore, activation of an immune response by the LPS challenge could have increased the mobilization of carotenoids, which have important immuno-modulatory functions, from gape tissues because of a trade-off in allocation of these limiting dietary components to the competing functions of gape coloration or immunity [43,66,67]. This interpretation is consistent with the results of previous studies showing a negative effect of an immune challenge on gape pigmentation of barn swallow nestlings [13,22].

Female, but not male, LPS nestlings showed significantly higher postural begging scores than control nestlings, irrespective of food deprivation. These intense begging solicitations of LPS females were probably caused by their impaired condition, as reflected by negative effects of LPS on other traits. Similar results were found in a previous study of begging in nestling great tits (*Parus major*) experimentally infested by ectoparasites, with infested broods increasing their begging rate [15]. Interestingly, control females did not significantly increase their solicitation displays after food deprivation. Females have been shown to be less susceptible to food shortage than males [25], and for this reason have been hypothesized not to escalate their begging output when needy siblings are also present [25]. Thus, this finding might corroborate previous finding of state sensitivity and favoritism towards needy kin in this species [25,26,28,49]. On the other hand, control males begged as vigorously as their LPS male siblings, probably because male barn swallow chicks compete for food more harshly than female chicks [25,49].

Parents did not respond to increased postural begging of LPS females by preferentially feeding them, as assessed both by parental feeding rates and mass gain during feeding trials, possibly because they relied on other components of begging, like gape coloration, besides postural displays. In fact, LPS females had less saturated palate and flange coloration than controls of the same sex. Duller gape coloration may thus have compensated for the effect of increased postural begging on parental decisions. However, this interpretation does not apply to LPS males which had less saturated palate coloration but did not beg more intensely than controls.

An alternative interpretation is that the negative effects of the immune challenge on condition may be transient [17,18,41,42,44] and not strong enough to justify a parental favoritism towards specific nestlings. In fact, we recorded just two episodes of mortality out of 102 LPS nestlings, which corresponds to baseline mortality among barn swallow nestlings under natural conditions [37]. This interpretation implies that parents can assess whether deterioration in offspring condition is ephemeral thus avoiding sacrifice of chicks whose state can easily be improved by a relatively small additional investment, and would be generally consistent with the observation that the barn swallow is a species adopting a 'brood-survival' strategy [37,40].

In conclusion, we showed that exposure to a Gram-negative bacterial endotoxin has diverse, detrimental effects on growth and begging behavior of barn swallow chicks. These effects were more evident among female chicks, disclosing an important role of bacterial infection during early life in determining sex-related differential growth and condition. Our findings further suggest that parasite infection during critical phases of feather growth, like early development or moulting, might be regarded as a cause of variation in feather quality.

Supporting Information

Table S1 Effects of LPS challenge on hue, chroma and brightness of gape and flanges. Table shows final mixed models reporting the effects of treatment, sex and their interaction (where statistically significant) on hue, chroma and brightness of palate and flanges of barn swallows nestlings at both day 2 and 3 after LPS injection.
(PDF)

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Author Contributions

Conceived and designed the experiments: AR NS DR GB. Performed the experiments: AR MC. Analyzed the data: AR RA DR. Contributed reagents/materials/analysis tools: NS. Wrote the paper: AR DR GB NS.

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SUPPORTING INFORMATION

Table S1. Effect of LPS on hue, chroma and brightness of gape and flanges. Final models reporting effect of treatment, sex and their interaction (where significant) on hue, chroma and brightness of palate and flanges of nestlings. All the analyses were performed both 2 and 3 days after the experimental manipulation. See Methods for details.

	Coefficient	χ^2	*df*	*P*
Hue palate (day 14) (n = 148)				
Treatment	-0.033 (0.020)	2.66	1	0.103
Sex	-0.036 (0.023)	2.39	1	0.122
Hue palate (day 15) (n = 146)				
Treatment	-0.010 (0.018)	0.33	1	0.568
Sex	0.038 (0.021)	3.36	1	0.067
Hue flange (day 14) (n = 148)				
Treatment	-0.052 (0.036)	2.17	1	0.141
Sex	-0.101 (0.041)	6.30	1	0.012
Hue flange (day 15) (n = 146)				
Treatment	-0.053 (0.038)	2.01	1	0.156
Sex	-0.010 (0.042)	0.05	1	0.815
Chroma palate (day 14) (n = 148)				
Treatment	-0.015 (0.006)	6.28	1	0.012
Sex	0.002 (0.007)	0.12	1	0.733
Chroma palate (day 15) (n = 146)				
Treatment	-0.008 (0.005)	2.23	1	0.135
Sex	0.011 (0.006)	3.47	1	0.062
Chroma flange (day 14) (n = 148)				
Treatment	-0.016 (0.007)	5.59	1	0.018
Sex	-0.020 (0.065)	7.95	1	0.005
Treatment × Sex	0.0023 (0.009)	6.03	1	0.014
Chroma flange (day 15) (n = 146)				
Treatment	0.004 (0.006)	0.43	1	0.512
Sex	-0.009 (0.006)	2.03	1	0.154
Brightness palate (day 14) (n = 148)				
Treatment	-0.011 (0.008)	2.11	1	0.147
Sex	-0.006 (0.009)	0.44	1	0.506
Brightness palate (day 15) (n = 146)				
Treatment	0.006 (0.007)	0.93	1	0.335
Sex	0.002 (0.008)	0.003	1	0.855
Brightness flange (day 14) (n = 148)				
Treatment	0.000 (0.001)	2.18	1	0.996
Sex	0.013 (0.015)	0.74	1	0.391
Brightness flange (day 15) (n = 146)				
Treatment	-0.008 (0.016)	0.29	1	0.590
Sex	0.031 (0.029)	2.98	1	0.084

PART II

SEX-RELATED PARENTAL INVESTMENT AND ECOLOGICAL CONDITIONS

Chapter 4

Secondary sex ratio covaries with demographic trends and ecological conditions in the barn swallow

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ORIGINAL PAPER

Secondary sex ratio covaries with demographic trends and ecological conditions in the barn swallow

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Abstract Parents are expected to invest more in the sex that benefits most from the local environment. When the quality of breeding sites varies spatially and natal dispersal of males and females differs, parents in high-quality habitats should skew their progeny sex ratio in favor of the less dispersing sex. We tested this prediction in the barn swallow (*Hirundo rustica* L.), by relating the proportion of male offspring around fledging (secondary sex ratio) of first and second broods to the ecological quality (presence of livestock farming and relative surface of hayfields in the foraging range) and local demographic trends of the farms where the colonies were located. Consistent with our predictions, the proportion of male offspring, which are more philopatric than females, increased with the extent of hayfields, which are high quality, preferred foraging habitats. Moreover, the proportion of male offspring in second broods was smaller in colonies with positive demographic trends, possibly indicating density-dependent effects on sex ratio. Independent of the mechanism generating uneven sex ratio (zygote sex ratio adjustment or sex-related pre-fledging mortality), barn swallows breeding under favorable conditions overproduced the sex that is more likely to benefit from such conditions.

Keywords Sex allocation · Natal dispersal · Demographic trend · Habitat quality · Julliard's model

Introduction

In sexually reproducing species, frequency-dependent selection should favor the production of a balanced offspring sex ratio, as this is the evolutionarily stable strategy whenever the expected reproductive value of offspring of either sex is proportional to parental

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investment (Fisher 1930; Frank 1990). On the other hand, sex allocation theory predicts that whenever the relationship between offspring reproductive value and parental investment is nonlinear, parents should bias their progeny sex ratio towards the sex that provides the higher fitness gain (Trivers and Willard 1973; Charnov 1982; Frank 1990). Although strong evidence exists for *adaptive* sex ratio adjustment in haplodiploid species (Godfray 1994), the frequency and occurrence of this adjustment is still controversial in vertebrates with chromosomal sex determination (West and Sheldon 2002; Ewen et al. 2004; Cassey et al. 2006). Several studies have provided evidence for non-random sex allocation in mammals (Clutton-Brock et al. 1984; Cameron 2004) and birds (Komdeur et al. 1997; Kilner 1998; Saino et al. 2010; see Hasselquist and Kempenaers 2002; Komdeur and Pen 2002 for reviews) and have also identified potential mechanisms for primary and secondary sex ratio manipulation (Correa et al. 2005; Lessells 1998; Badyaev et al. 2002; Young and Badyaev 2004; see Oddie 1998; Hasselquist and Kempenaers 2002 for reviews).

Several studies have disclosed patterns of sex ratio variation consistent with expectations from theoretical models. For example, when maternal condition at the time of reproduction is good or when food availability is high, progeny sex ratio is biased towards the more costly sex (Bradbury and Blakey 1998; Kilner 1998; Nager et al. 1999). Additionally, dispersal ability has also been related to sex allocation strategies (Komdeur et al. 1997; Forero et al. 2002), particularly in birds where females usually disperse farther than males (Greenwood 1980; Greenwood and Harvey 1982). Theoretical models predict that in species with sex-biased natal dispersal parents should produce a skewed offspring sex ratio in response to the environmental conditions at the breeding site. When the risk of inbreeding is high (e.g. when the population is small or isolated; Martín et al. 2008) parents may benefit from producing an offspring sex ratio biased towards the sex that disperses more ("inbreeding avoidance hypothesis"; Greenwood and Harvey 1982; Pusey 1987; Pusey and Wolf 1996). Furthermore, when local competition for resources is intense, parents should overproduce the more dispersing sex in order to reduce the competitive interactions among their offspring ("local resources competition hypothesis"; Clark 1978; Silk 1984; Gowaty 1993). Julliard (2000) proposed a theoretical model of optimal sex ratio in species with sex-biased natal dispersal in heterogeneous breeding environments. This model predicts that parents should invest more in the sex that benefits the most from the local environment. Thus, parents are expected to bias reproduction towards the more philopatric sex in good-quality habitats and, conversely, favor the more dispersing sex where ecological conditions are poor (Julliard 2000).

The main aim of this study was to test whether offspring 'secondary' sex ratio (i.e. the sex ratio recorded around fledging) covaries with breeding habitat quality. We used the barn swallow (*Hirundo rustica* L.) as our model system since males are more philopatric than females (Møller 1994; Balbontìn et al. 2009) and conditions are thus met for testing whether parents produce more sons in good habitats compared to breeding sites in poor conditions.

To this end, we assessed the quality of the breeding habitat during the year of the study using two variables known to affect distribution, abundance and foraging activity of the barn swallow: the presence of dairy farming and the extent of hayfields around the colony (Ambrosini et al. 2002). Additionally, because long-term (relative to barn swallow lifespan) changes in the number of breeding pairs within a colony may also reflect variation in local habitat conditions, we quantified the proportional variation in the number of breeding pairs at individual colonies (see "Methods"). As sex allocation strategies and sex-related pre-fledging mortality may vary depending on seasonal and local ecological effects as well

as previous reproductive effort of parents, we analyzed variation in sex ratio in the first and second broods. We also investigated offspring sex ratio in relation to the mean reproductive success of first and second broods at each colony, because parents should favor the production of the less dispersing sex in habitats where they can afford higher reproductive success (Julliard 2000). Finally, we analyzed variation in fledging success according to the proxies of habitat quality as well as to colony size, in order to test for possible density-dependent effects.

We predicted that the abundance of hayfields and the presence of cattle positively affected the proportion of male offspring. We did not have a specific prediction on the sign of the covariation of offspring sex ratio with demographic trend. On one hand, it could be argued that the number of breeding pairs increased in habitats with optimum ecological conditions and, therefore, the sex ratio should be biased in favor of the more philopatric sex. On the other hand, an increase in number of breeding pairs may increase local competition, thus favoring the production of the more dispersive sex. Finally, we expected that sex ratio was more male-biased in colonies with relatively large fledging success.

Materials and methods

Study organism

The barn swallow is a small, insectivorous, semi-colonial, socially monogamous migratory passerine with sex-biased natal dispersal (Møller 1994) that varies considerably among populations (Balbontìn et al. 2009). In our study population, natal dispersal is high, and recruitment rate at the natal site is low and biased towards males (Saino et al. 2002). Indeed, during intensive studies conducted in 1993–2008 in the same geographical region where this study was carried out, 205 (84%) out of the 245 nestlings recaptured as breeding adults in their natal colony were males. Adults show strong breeding site fidelity (Møller 1994; Saino et al. 1999). Life expectancy at hatching is about 2 years due to a mortality rate of about 60% each year (Møller 1994) whereas the maximum recorded lifespan in our study area is 7 years (N. Saino, unpublished data). Females lay 1–3 clutches of 1–7 eggs (modal size: 5 eggs) per breeding season (Møller 1994). Nestlings hatch asynchronously approximately 14 days after the start of incubation, and both parents provide food. Nestlings fledge when they are 18–20 days old (Møller 1994).

Field methods and molecular sexing

The study was carried out in April–August 2008 at 64 colonies (=farms) located in the Parco Regionale Adda Sud (240 km^2; Northern Italy), an intensively cultivated lowland where livestock farming is widespread (Fig. 1; see Ambrosini et al. 2002 for a detailed description of the study area). In this area, annual censuses of breeding swallows have been performed since 1999 in 99–165 farms (see also Ambrosini et al. 2002 for details). Briefly, for the purposes of this study we visited all farms once every 7–14 days. During each visit, we inspected all nests and recorded the number of eggs and nestlings. We estimated nestling age according to body size and plumage development (see Ambrosini and Saino 2010 for a validation of this method). We then estimated hatching date of each brood according to nestling age, while laying date was calculated assuming that barn swallows lay one egg per day and incubate eggs for about 14 days (Møller 1994; Ferrari et al. 2006). We expressed fledging success as the proportion of the eggs that generated a chick

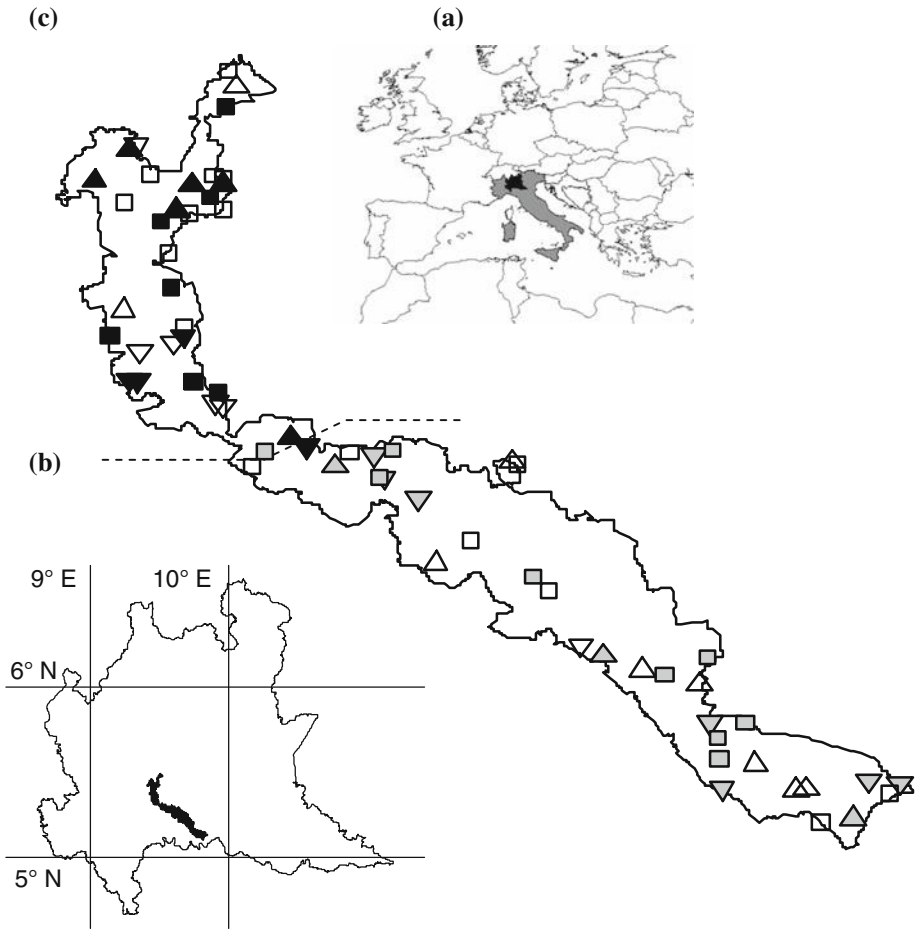

Fig. 1 **a** Lombardy in Italy and Europe. **b** The study area in Lombardy. **c** Location of monitored colonies within the study area. The *shape* and *color of symbols* indicate demographic trends and study procedure, respectively (*point up triangles* increasing colonies; *squares* stable colonies; *point down triangles* declining colonies; *black symbols* colonies where both first and second broods were sexed; *gray symbols* colonies where only first broods were sexed; *white symbols* colonies where no broods were sexed). In the farms above the dashed line both first and second broods were monitored for estimating fledging success

surviving at least to the age of 10 days. Mortality after age 10 days accounts for less than 5% of total nestling mortality (N. Saino, unpublished data). We expressed mean within-colony fledging success as the mean of fledging successes of each brood. The maximum number of nests simultaneously active (i.e. with eggs or nestlings) in each farm was used to estimate the number of breeding pairs in that colony (see Ambrosini et al. 2002).

Owing to practical limitations in performing extensive field work during the whole breeding season, we choose to record data of second broods only in 34 farms located in the northern region of the study area (Fig. 1), in 30 of which second broods were laid.

A sample of 36 out of the 64 monitored colonies was chosen for collection of data on secondary sex ratio according to their demographic trends in the five breeding seasons preceding that of the study (i.e. 2003–2007; Fig. 1). Because farms that have been only

recently colonized may show an increasing demographic trend independently of habitat quality, potentially confounding the correlation between population trend and habitat variables, each farm included in this sample hosted a barn swallow colony at least since 1999, the year when an extensive census in our study area started (Ambrosini et al. 2002, 2006). We estimated demographic trends by calculating the proportional variation of breeding pairs during the 5-years time series (λ parameter in a geometric population growth model, where $\lambda = 1 + r$, the population growth rate; see below). We a priori decided to use demographic trends computed since 2003 (5 years before the year of collection of sex ratio data) because trend estimates calculated over longer time-spans may be affected by long-term trends in barn swallow populations, which have been shown to be negative at large, continental scale (BirdLife International 2004), and may thus not accurately reflect short-term variation in local conditions at individual colony sites. On the other hand, trends calculated over only a few recent years may not be accurate because they could be strongly influenced by stochastic fluctuations in the number of breeding pairs. However, trends calculated assuming any other start year between 1999 and 2005 were significantly positively correlated with those computed between 2003 and 2007 (r values always ≥ 0.730; $n = 36$, $P < 0.001$). We considered as increasing or declining those colonies where population growth rate in 2003–2007 was larger than 0.1 or smaller than -0.1, respectively. The other colonies were considered stable ($-0.1 \leq r \leq +0.1$). Prior to the start of fieldwork, we selected twelve colonies for each category of demographic trend (i.e. declining, stable, or increasing) among the farms that have been monitored since 1999, according to practical considerations on the accessibility of nests. Before statistical analyses, we updated estimates of demographic trends including colony size in the study year (2008). After this procedure, some farms changed their category of demographic trend, resulting in 11 declining, 16 stable and 9 increasing colonies. 17 of these colonies (4 declining, 7 stable, 6 increasing) belonged to the subset of farms where we also collected data on second broods (see above; Fig. 1).

Given the large number of colonies included in this study, the impossibility of performing mist-netting at several farms/stables, and of identifying the composition of breeding pairs by focal observations, we only identified second broods as all broods laid after fledging of the nestlings that had hatched first in each colony (Ambrosini et al. 2002, 2006). The very few (18) third broods identified with the same criterion were discarded. In the analysis of sex ratio we only included second broods that were selected with a more restrictive criterion: we considered only second broods laid in the same nest where a first brood fledged successfully. Given that consecutive broods in the same nest are laid by the same female in the vast majority of cases, and that pairs seldom divorce during a single breeding season (Møller 1994), this procedure allowed us to obtain information on sex ratio only for those broods that were very likely second broods (rather than first broods laid late in the season). The other second broods identified with the criterion described above were only included in the analysis of fledging success, together with all the second broods of the colonies where sex ratio data were not collected.

In the barn swallow, nestlings cannot be sexed based on external phenotype (Møller 1994) and we therefore applied molecular sexing techniques (see below) on blood samples collected from nestlings of age 10 days or older. Blood samples were collected by puncturing the brachial vein and stored in a cool bag in the field and then at -20°C in the lab for later molecular analyses. Since in our study population nestling mortality after age 10 days (excluding nest destruction and predation) is lower than 5% of total nestling mortality (N. Saino, unpublished data), this method allowed us to obtain a good estimate of brood sex ratio close to fledging (secondary sex ratio). We focused on offspring secondary

sex ratio because we aimed at assessing whether the proportion of male nestlings covaried with environmental quality, independently of the specific mechanisms (e.g. primary sex ratio bias, sex-related embryo or post-hatching mortality) that may be causing any variation in sex ratio.

We stress that collecting information on primary sex ratio (i.e. sex ratio of fertilized eggs) was impractical in this study, as it is likely to be in any large-scale study of free ranging birds for two reasons. First, sexing of fertilized eggs that fail to hatch is often impossible because of early embryo mortality, which prevents collection of embryonic materials several days later and the fact that addled eggs are removed from the nest by parents. Secondly, for ethical and practical reasons blood sampling should be avoided in the first days after hatching, when some mortality occurs, and most of the dead nestlings are removed from the nest.

We performed molecular sexing on all the first broods in the 36 farms of the sample, except for those that were inaccessible (e.g. because of presence of dangerous animals like bulls). Sex ratio was also recorded for all the second broods reared in the same nest of a sexed first brood in the 17 farms where second broods were monitored.

We performed molecular sexing of nestlings following the protocol originally developed by Griffiths et al. (1998), slightly modified according to Saino et al. (2008b).

Environmental conditions

We recorded the proportion of area occupied by different types of land use in a radius of 200 m around each farm. This distance was chosen as observations made in the same study area indicated that most individuals forage within this distance from the colony (Ambrosini et al. 2002). In addition, preliminary analyses suggest that the surface of hayfields within this radius predicts colony size (R. Ambrosini, unpublished results). We assessed land use by direct observation and reported it on detailed maps (scale: 1:10,000). In particular, we focused on the extent of hayfields around farms because barn swallows show a preference for this type of crop when foraging (Ambrosini et al. 2002), as the density of individuals foraging on hayfields is approximately 13 times larger than on maize, which is the other prevailing crop in the area (Ambrosini et al. 2002).

Since it has been repeatedly shown that dairy farming affects presence, abundance and reproduction of barn swallows (Møller 2001; Ambrosini et al. 2002, 2006; Ambrosini and Saino 2010; Grüebler et al. 2010), we also recorded the presence of cattle in the farm. These data were used to generate a dichotomous variable representing cattle rearing in the year of the study.

Statistical methods

We calculated trends in colony size as the slope of the regression of log-transformed number of breeding pairs on time, corresponding to the λ parameter in models of geometric population growth models (Eberhardt 2007). Sex ratio (proportion of males of males relative to the number of nestlings fledged in a nest) and fledging success (proportion of nestling fledged relative to clutch size) of first and second broods were then analyzed in binomial Generalized Linear Mixed Models (GLMMs) that accounted for data overdispersion. These models included as predictors the demographic trend (three-levels factor, see above), a dichotomous factor indicating first or second broods (hereafter "brood"), a dichotomous factor indicating presence or absence of dairy farming in the farm, proportion of hayfields (continuous covariate), and all two-way interactions among these fixed effects

and brood. In the analysis of sex ratio we also included mean within-colony fledging success as predictor. In the analysis of fledging success we also included colony size as well as the interaction between colony size and brood to account for density-dependent effect on nestling survival. Farm identity and nest identity, nested within farm, were included as random factors. Degrees of freedom were estimated according to the Kenward-Roger approximation and final models were obtained by removing non-significant predictors from the initial model.

Following Nakagawa (2004) we did not use any multiple testing correction for the P values in post hoc tests (e.g. Bonferroni correction). All the analyses were run in SAS 9.1 (SAS Institute Inc., Cary USA) with the GLIMMIX procedure.

Results

Sex ratio (number of male nestlings/brood size at age of blood sampling) data were available for 329 first broods in 36 farms (total sample = 1,456 nestlings) and 65 s broods in 17 farms (258 nestlings) and fledging success data were available for 465 first broods in 64 farms and 187 s broods in 30 farms. In the same 36 farms where we recorded brood sex ratio, we also recorded fledging success of 418 first and 183 s broods. The difference in sample size compared to sex ratio data is justified by the inclusion in the current analysis of broods where all nestlings died before we could take blood samples and of clutches where all eggs failed to hatch, and by the restrictive criterion used to identify second broods to include in the sex ratio analyses (see "Methods").

Sex ratio of first broods was 0.503 ± 0.013 SD while that of second broods was 0.461 ± 0.031 SD. Sex ratios of both first and second broods were not significantly different from 0.5 (binomial exact test: $P \geq 0.237$ in both cases), nor did they differ from each other ($\chi^2_1 = 1.349$; $P = 0.246$). Trends in colony sizes calculated since 2003 ranged from −0.320 to 0.268 across the 36 farms where sex ratio in first broods was investigated (mean = −0.006 ± 0.024 SE; median = 0.007) and from −0.320 to 0.215 in farms where also second broods were sampled (mean = 0.037 ± 0.034 SE; median = 0.089).

A binomial GLMM of sex ratios including population trend (three-levels factor), brood, their interaction, and the proportion of hayfields within 200 m of the colony is shown in Table 1. The coefficients of the model indicated that sex ratio significantly increased with the proportion of hayfields around the colony (Fig. 2; Table 1), and it was not predicted by the presence of dairy farming (coefficient: −0.0003 ± 0.0004 SE; $F_{1,355.7} = 0.57$; $P = 0.451$) or fledging success (coefficient: −0.129 ± 0.400 SE; $F_{1,382.7} = 0.10$; $P = 0.748$). The statistical effect of population trend on sex ratios differed between first and second broods (Table 1). Sex ratio did not show a clear variation in relation to demographic trend in first broods, while in second broods it declined (i.e. more female-biased) at increasing levels of colony growth rates (Fig. 3). However, post hoc tests showed that the sex ratio of first broods was significantly more male-biased in stable than in declining colonies (estimated difference = −0.311 ± 0.145; $t_{373.7} = -2.14$; $P = 0.033$; see also Fig. 3 for observed values). In addition, in declining colonies sex ratio of second broods was significantly more male-biased than that of first broods (estimated difference = 0.773 ± 0.352; $t_{278.6} = -2.20$; $P = 0.029$), while the reverse held true in increasing colonies (estimated difference = 0.467 ± 0.194; $t_{182.3} = 2.41$; $P = 0.017$; Fig. 3). Finally, second broods in declining colonies hosted more male nestlings than second broods in increasing colonies (estimated difference = 1.038 ± 0.374; $t_{374.4} = 2.78$; $P = 0.006$), indicating a decreasing

Table 1 General linear mixed models (GLMM) analysis of sex ratio and fledging success

Effect	F	*df*	*P*
Sex ratio			
Population trend	2.55	2, 332.8	0.080
Brood	0.04	1, 261.2	0.833
Population trend × Brood	4.77	2, 263.8	0.009
Hayfields proportion[a]	4.18	1, 345.5	0.042
Fledging success			
Brood	49.20	1, 534.6	<0.001
Colony size	10.03	1, 39.06	0.003
Colony size × Brood	28.71	1, 524.4	<0.001

The analyses of sex ratio were conducted on a sample of 329 first broods in 36 farms and 65 s broods in 17 farms. The analyses of fledging success was performed on a sample of 465 first broods in 64 farms and 187 s broods in 30 farms. Only final models are presented here

[a] Estimate ± SE: 1.163 ± 0.772

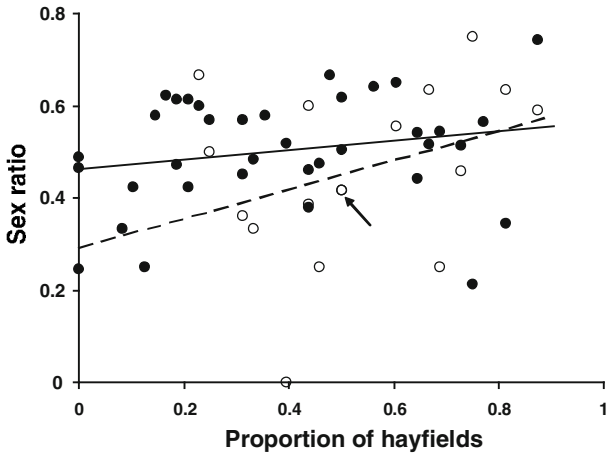

Fig. 2 Observed mean brood sex ratio (=proportion of male offspring) in relation to the proportion of hayfields within 200 m of the colonies. *Black dots* first broods in 36 farms; *white dots* second broods in 17 farms. No significant effect of interaction between hayfield and brood was found ($F_{1,304.1} = 0.98$; $P = 0.323$). However, regression lines (*filled line* first brood; *dashed line* second brood) fitted to the mean within-farm sex ratio data of first or second broods are presented for completeness and clarity. The *arrow* indicates two overlapping data points

sex ratio in colonies with increasing demographic trend. The other pair-wise comparisons were not significant (Fig. 3).

Fledging success was lower in first than in second broods (estimated difference = −1.599 ± 0.215; $t_{530.9} = -7.45$; $P < 0.001$) and differently varied with the number of breeding pairs in first and second broods, as indicated by the significance of the interaction between colony size and brood (Table 1). In particular, the fledging success in second broods was severely affected by the number of breeding pairs (coefficient: −0.083 ± 0.019 SE; $t_{60.29} = -4.33$; $P < 0.0001$), while it did not covary with colony size in first broods (coefficient: −0.025 ± 0.016 SE; $t_{35.21} = -1.49$; $P = 0.146$). The final model also indicated that fledging success was not affected by dairy farming (coefficient:

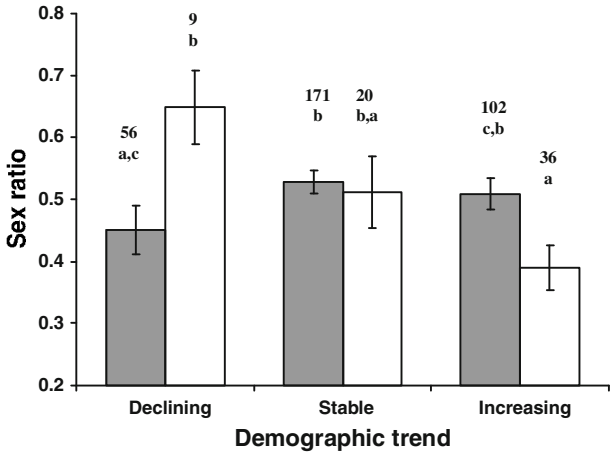

Fig. 3 Observed mean brood sex ratio (proportion of male offspring) of first (*filled columns*) and second broods (*empty columns*) in farms with different population trends. *Bars* represent standard errors; *numbers* indicate the number of clutches in the sample. *Different letters* indicate significant difference ($P < 0.05$) between groups, calculated from the final model

0.0006 ± 0.014 SE; $F_{1,52.12} = 0.18$; $P = 0.671$), by the proportion of hayfields (coefficient: 1.163 ± 0.773 SE; $F_{1,50.73} = 2.27$; $P = 0.138$) and by the local demographic trend ($F_{2,53.78} = 1.34$; $P = 0.271$).

Finally, we found no significant difference between sex ratio of first and second broods laid by the same pairs (difference = −0.056 ± 0.041 SE; $Z = 1.311$; $P = 0.190$), and there was no significant correlation between the sex ratio recorded in the first broods and that in the corresponding second broods (Spearman's rank correlation: $\rho = 0.214$; $P = 0.100$, $n = 60$).

Discussion

In the present study, we found that the proportion of male offspring increased with the area occupied by hayfields, which are the preferred foraging habitats of barn swallows. However, the proportion of male offspring did not depend on the presence of cattle farming, which is known to affect the distribution and abundance of breeding barn swallows (Møller 2001; Ambrosini et al. 2002). In addition, the proportion of males in second broods was smaller in colonies that had increased in size while sex ratio did not covary with average fledging success within colonies. Finally, fledging success was biased towards males in second compared to first broods and declined with colony size only in second broods.

Animal farming and agricultural land use are well known factors affecting the distribution and abundance of breeding swallows (Møller 2001; Ambrosini et al. 2002; Grüebler et al. 2010). Hayfields and pastures are the preferred foraging habitats for the aerially insectivorous barn swallows, as the density of individuals foraging in these habitats exceeds by one order of magnitude that in the other prevailing crops (e.g. maize; Ambrosini et al. 2002). Overproduction of males in areas with relatively large pastures and hayfields may thus represent an adaptive sex allocation strategy, because males are the more philopatric sex and are thus more likely to benefit from favorable local environmental conditions. Conversely, a female-biased offspring sex ratio should be favored in colonies

with relatively fewer surrounding hayfields. Thus, the observed variation in sex ratio was consistent with these predictions. Producing relatively more offspring of the more dispersing sex when breeding in a poor-quality habitat is advantageous particularly in species with extremely high breeding philopatry, like the barn swallow (Møller 1994; Turner 2006) because natal dispersal is the only chance of escaping low-quality habitats for breeding. Hence, these results support the hypothesis of differential sex allocation strategies favoring the production of the more dispersing sex in poor quality habitats (Julliard 2000), even though sex ratio variation was not influenced by the mean fledging success within a colony.

The lack of effect of reproductive success on the observed sex ratio is not surprising, because in our model species brood size and fledging success are not influenced by the ecological variables that affect the distribution and the local abundance of barn swallows (i.e. the presence of cattle; Ambrosini et al. 2006; Ambrosini and Saino 2010). Furthermore, the small variation in fledging success in our study population reduced the scope for significant relationships with habitat features.

An alternative interpretation is that the pattern of sex allocation observed is coincidental with the effect of ecological conditions on pre-fledging survival of sons and daughters. Previous studies of the same population have shown that male offspring are competitively superior to their sisters when scrambling for parental care (Bonisoli-Alquati et al. 2011) but that they are more susceptible to prolonged adverse conditions such as those imposed by being in a large brood or after a period of food deprivation (Boncoraglio et al. 2008, Saino et al. 2008a). Hence, males are more likely to benefit from being reared under favorable habitat conditions and this may explain the larger proportion of sons raised in farms surrounded by large hayfields. Biases in sex ratios may thus result from sex-related variation in mortality, rather than from adaptive strategies of production of zygotes of either sex.

The mechanisms generating the observed pattern of sex ratio variation are unknown. However, it seems unlikely that parental favoritism towards offspring of either sex had caused sex-related mortality, based on previous observations from the same population (Saino et al. 2002) and by the general observation that the barn swallow adopts a brood survival strategy (Møller 1994; Turner 2006). Independent of the mechanism, our study shows that, under favorable conditions, the proportion of nestling fledged is skewed towards the sex most likely to benefit from such conditions.

The only direct test of adaptive sex allocation via manipulation of primary (zygote) sex ratio is obviously unfeasible in an extensive study like the present one because it would entail the ethically unacceptable sacrifice of a large number of eggs. Even an extensive sampling of chicks immediately after hatching, in order to assess the sex ratio before post-hatching mortality, is highly unpractical on several hundreds of chicks. Data on offspring sex ratio may therefore reflect the effects of any sex allocation strategy adopted by parents in conjunction with the effects of ecological conditions on survival of either sex. It should be noted that even considering broods where no mortality occurred would not be an appropriate way of testing for 'primary' sex allocation strategies (Fiala 1980; Krackow and Neuhäser 2008). This is the case because sex ratio in broods where all nestlings survive may not be representative of the overall sex ratio at the local population (i.e. colony) level if offspring mortality differed between sexes.

Present results are also consistent with the classic Trivers and Willard (1973) hypothesis of parents producing a progeny whose sex ratio is biased toward the more costly sex when rearing conditions are good (e.g. when food availability is high; Kilner 1998). In our model species, male and female nestlings are monomorphic and do not differ in phenotypic traits until the first molt (Saino et al. 2002). However, sons suffer more from chronic food deprivation compared to daughters (Boncoraglio et al. 2008; Saino et al. 2008a), causing

parents to increase their feeding efforts (Saino et al. 2000; Boncoraglio et al. 2008). Hence, under favorable foraging regimes, the conditions are met for parents to invest more in high-quality sons because they are expected to produce more offspring than high-quality daughters due to variation in mating success and sperm competition (Møller 1994; Turner 2006). This implies that an extra investment in high-quality sons should be advantageous, because they are expected to produce more offspring than high-quality daughters.

We did not find a statistically significant effect of population trend on sex ratio in the first broods. However, we found a significant interaction between brood and population trend, with offspring sex ratio in the second broods significantly covaried negatively with local population trend. Whether a local increase in colony size entails a larger competition compared to more declining colonies or, at the opposite, allows for a 'competitive release', depends on whether the increase in the number of competitors significantly outweighs local improvement in ecological conditions. The effects of local population dynamics on competition, however, cannot be estimated in the present study. If positive proportional changes in colony size entail an increase in competition, present results may suggest that density-dependent effects on sex ratio will become effective in second broods, when competition for food may be more intense (Møller 1994; Turner 2006). This interpretation is corroborated by the negative effect of colony size on fledging success in second rather than first broods. The sex ratio variation pattern observed in the second broods may thus be caused by a larger parental investment towards females, the more dispersive sex, in colonies where competition has become more intense as a consequence of a recent increase in size ("local resource competition hypothesis"; Clark 1978; Silk 1984; Gowaty 1993), or could result from a male-biased mortality in second broods. In the first case, larger production of daughters may function to compensate for habitat selection that becomes inappropriate at the time when second broods are reared. In the second scenario, it could be argued that in colonies whose size has recently increased, more intense competition caused larger mortality of the sex (male) that is more susceptible to harsh rearing conditions (Boncoraglio et al. 2008; Saino et al. 2008a). Because trends in colony size depend on recruitment of yearlings and not on breeding dispersal, and relatively old females produce more daughters (Saino et al. 2002), an alternative interpretation is that a female-biased sex ratio in declining colonies may result from relatively large age of breeding females.

In conclusion, this study has disclosed patterns of secondary sex ratio variation in relation to ecological conditions of the breeding site. However, no firmly conclusions can be drawn about the mechanisms that generate such observed variation (parental sex allocation strategies or sex-related pre-fledging mortality). Future studies combining information on sex allocation, natal philopatry and ecological conditions at different spatial and temporal scales will allow for tests on the generality of the patterns of sex ratio adjustment documented here for the barn swallow.

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Chapter 5

Seasonal decline of offspring quality in the European starling *Sturnus vulgaris*: an immune challenge experiment

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ORIGINAL PAPER

Seasonal decline of offspring quality in the European starling *Sturnus vulgaris*: an immune challenge experiment

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Abstract In seasonally fluctuating environments, timing of reproduction is a crucial determinant of fitness. Studies of birds show that late breeding attempts generally result in offspring of lower reproductive value, with lower recruitment and long-term survival prospects. Several proximate mechanisms, including a seasonal decline of immune system functioning, may lead to a seasonal decline of offspring fitness. We investigated seasonal variation in offspring quality by subjecting first- and second-brood chicks of a sexually size dimorphic species, the European starling *Sturnus vulgaris*, to an immune challenge with a bacterial endotoxin (LPS), and evaluated their growth and physiological response in terms of total plasma antioxidant capacity (TAC), concentration of reactive oxygen metabolites and hematocrit. LPS challenge did not affect chick growth or oxidative status. However, hematocrit of second-brood chicks was higher in LPS chicks compared to controls. Body mass halfway through the rearing period (days 8–9 post-hatching), TAC and hematocrit were lower among second- vs. first-brood chicks. Interestingly, sexual dimorphism in body mass at days 8–9 post-hatching markedly differed between broods, first-brood males being 4.7% and second-brood males 22.7% heavier than their sisters, respectively. Pre-fledging mortality occurred among second-brood chicks only and was strongly female-biased. Our findings suggest that starling chicks, even if in poor conditions, are little affected by a bacterial challenge, at least in the short-term. Moreover, our study indicates that sex differences in body size, possibly mediated by sex-specific maternal investment in egg size, may heavily impact on pre-fledging survival in a different way in the course of the breeding season, resulting in sex-specific seasonal decline of offspring fitness. Finally, we suggest that levels of circulating antioxidants should be regarded among the proximate causes of the association between timing of fledging and long-term survival in avian species.

Keywords Antioxidants · Immune challenge · LPS · Oxidative stress · Seasonal variation · Sex-biased investment

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Introduction

In seasonal environments, where ecological resources fluctuate, timing of reproduction is a major determinant of reproductive performance and fitness, since individuals that breed at the time of peak resource availability achieve greater fitness (Clutton-Brock 1988; Iwasa and Levin 1995). Natural selection on timing of breeding, acting via selection on the offspring, is therefore expected to be intense, and the fitness consequences of variation in timing of reproduction have been the subject of many studies, especially on birds, starting from the pioneering work by Lack (1947, 1954) (see also Klomp 1970; Perrins 1970). These studies mainly concern the relationships between fitness components, such as clutch size, fledging success, or survival/recruitment, and laying date, with some documenting hump-shaped relationships between fitness and laying date, while others highlighting linear declines of reproductive performance as the season progresses (e.g., Crick et al. 1993; Naef-Daenzer et al. 2001; Grüebler and Naef-Daenzer 2010). Whatever the shape of the seasonal fitness curve, there is general consensus that late nesting attempts result in low fitness returns (Crick et al. 1993). Two main ecological mechanisms have been advocated to explain a seasonal decline of reproductive success (review in Verhulst and Nilsson 2008). First, the 'parental quality hypothesis' posits that the low breeding output of late breeding individuals derives mainly from low-quality parents (e.g. younger and less experienced breeders, or parasitized birds) reproducing later (e.g. Rowe et al. 1994; Brinkhof et al. 1997; Møller et al. 2004). On the other hand, the 'breeding date hypothesis' posits that low breeding output of late breeding individuals is a consequence of a seasonal deterioration of environmental conditions, resulting in poor foraging success and thus nutritional constraints on offspring growth and condition (e.g. Parsons 1975; Brinkhof et al. 1993; Verboven and Verhulst 1996; Grüebler and Naef-Daenzer 2008). The two mechanisms are not mutually exclusive and may concur (e.g. parental body condition may decline in the course of the breeding season because resources deteriorates and this may in turn affect offspring fitness) to determine a seasonal decline in breeding performance and fitness, to the point that the two hypotheses are hardly distinguishable (Verhulst and Nilsson 2008). Moreover, genetic variation in timing of breeding may be maintained because seasonal clines in selection originate adaptive phenotypic clines, reinforced by assortative mating of early- and late-breeding individuals (Hendry and Day 2005). This may lead to reduced gene flow between early- and late-breeding individuals and genetic differentiation in relation to timing of breeding (Casagrande et al. 2006).

Many passerine birds lay two or more clutches per season (Cramp 1998) and thus offer the opportunity to investigate seasonal variation in offspring fitness among subsequent reproductive events within the same season while holding genetic variation in parental quality constant. Second clutches are often smaller (Cramp 1998) and produce low quality offspring with lower survival prospects (Hochachka 1990; Dubiec and Cichoñ 2001), though this effect may vary among years and species and depend on both pre- and post-fledging ecological conditions (Verboven and Visser 1998; Merino et al. 2000; Christe et al. 2001; Møller 2002; Grüebler and Naef-Daenzer 2008; López-Rull et al. 2011). In addition, offspring of some species may be sexually dimorphic already at the chick stage (Griffiths 1992; Badyaev 2002; Mainwaring et al. 2010), and male and female chicks may be differentially susceptible to seasonal deterioration of ecological conditions, either because of sex-specific susceptibility to harsh environments (Clutton-Brock et al. 1985; Griffiths 1992; Råberg et al. 2005; Bonisoli-Alquati et al. 2008) or to sex-related asymmetries in scrambling competition for access to parental resources (Uller 2006; Boncoraglio et al. 2008). In the first case, offspring of the larger sex (typically males) are predicted to achieve lower fitness, whereas in the second case, the opposite may be the case, as larger offspring may prevail in sibling competition vs. smaller offspring and enhance their share of food delivered by parents (Uller 2006).

Several, possibly interrelated, proximate mechanisms may account for the seasonal decline of offspring fitness. Although body size at fledging, partly reflecting nutritional conditions, positively predicted the probability of recruitment into the breeding population in studies of different passerine species (e.g. Hochachka and Smith 1991; Magrath 1991; Verboven and Visser 1998), hatching date was still found to predict recruitment irrespective of body size (Hochachka 1990; Verboven and Visser 1998). Moreover, several studies pointed out that immune responsiveness at the chick stage, implying a better ability to fend off parasites and pathogens, may also be an important predictor of long-term survival and recruitment, with even stronger effects than body size (Christe et al. 2001; Møller and Saino 2004; Moreno et al. 2005; López-Rull et al. 2011). Indeed, offspring immune responsiveness is a highly resource- and condition-dependent trait (Saino et al. 1997; Lochmiller and Deerenberg 2000; Norris and Evans 2000), and offspring in good immune conditions may thus survive better in the long term than those in poor immune conditions independently of body size per se (e.g. López-Rull et al. 2011). Several studies also reported that offspring immune responsiveness declines in the course of the breeding season, early-hatched offspring showing higher immunocompetence than late-hatched ones (Sorci et al. 1997; Dubiec and Cichoñ 2001; Wilk et al. 2006; López-Rull et al. 2011; but see Christe et al. 2001; Merino et al. 2000). Therefore, immune system functioning may qualify among the proximate factors causing a seasonal decline of offspring fitness.

In this study, we investigated seasonal variation of offspring quality in the sexually size dimorphic European starling (*Sturnus vulgaris*) by experimentally challenging the nestlings' immune system with a bacterial endotoxin (lypopolysaccharide (LPS) from *Escherichia coli* cell walls) and analysing their physiological and growth response, under the general expectation that high-quality nestlings in prime conditions should pay smaller costs for mounting an immune response compared to low-quality, poor condition, ones. We compared the effects of the immune challenge between first- and second-brood nestlings rather than early- and late-hatched ones, thereby controlling for variation in average genetic makeup of parents (see, e.g. Dubiec and Cichoñ 2001; Christe et al. 2001; Merino et al. 2000; López-Rull et al. 2011). LPS challenge has been often adopted to investigate the short-term effects of immune system activation by a bacterial endotoxin in the absence of the deleterious effects of the living pathogen (e.g. Bonneaud et al. 2003; Lee et al. 2005; Owen-Ashley et al. 2006; Romano et al. 2011). LPS is an inert, non-replicating antigen that induces a rapid inflammatory response, starting within a few hours after injection, triggering first a non-specific cell-mediated response that is followed by a humoral response and development of specific antibodies (Janeway and Travers 1999; Grindstaff et al. 2006).

As indicators of response to immune challenge, 24 h later (i.e. during the so-called acute phase response; Owen-Ashley and Wingfield 2007), we recorded total plasma antioxidant capacity (TAC), a measure of the overall capacity of tissues to resist attack by reactive oxygen species, plasma concentration of reactive oxygen metabolites (ROM), a marker of early oxidative damage (Costantini 2008; Monaghan et al. 2009), hematocrit and changes in growth. The innate inflammatory response induced by bacterial endotoxins does not come at no cost for the organism, as it is known to release free radicals that neutralise pathogens via their cytotoxic effects, but concomitantly damage molecules and cells (Halliwell and Gutteridge 1999; Bhattacharyya et al. 2004; Bertrand et al. 2006; Costantini 2008; Costantini and Møller 2009; Monaghan et al. 2009). These side effects of inflammation which may impose a limit to upregulation of the immune response are therefore expected to alter the oxidative status of the organism (Halliwell and Gutteridge 1999; Bhattacharyya et al. 2004; Bertrand et al. 2006; Costantini 2008; Costantini and Møller 2009; Monaghan et al. 2009). LPS is known to trigger secretion of pro-inflammatory cytokines by phagocytic cells that rapidly stimulate release of reactive oxygen and nitrogen species (Soszynski and Krajewska 2002; Bhattacharyya et al. 2004). Thus, LPS challenge may result in an increase of ROM and a decrease of TAC because circulating antioxidants (both endogenous and exogenous) may be used up to counter the side effects of the inflammatory response (Costantini 2008; Costantini and Møller 2009). Moreover, in birds, LPS is known to induce mass loss (Bonneaud et al. 2003; Bertrand et al. 2006) and to depress chick growth (Grindstaff 2008; Romano et al. 2011) either because of the direct energetic costs of mounting an inflammatory and immune response (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000) or of behavioural effects due to the induction of a characteristic 'sickness behaviour', resulting in reduced activity and food intake (Owen-Ashley et al. 2006). Finally, hematocrit, a measure of relative volume of erythrocytes over total blood volume, is a widely used condition index in avian studies, though its interpretation is not straightforward (review in Fair et al. 2007). However, both pathogen infection and changes in energetic condition and metabolism, such as those resulting from LPS challenge, may affect hematocrit values (Fair et al. 2007).

We predicted that second-brood chicks were in generally poorer condition than first-brood ones and therefore that the overall costs of the immune challenge (see Lochmiller and Deerenberg 2000) should be greater among second-brood chicks, resulting in slower growth among second- vs. first-brood LPS chicks compared to controls. We also formulated the general prediction that ROM should increase and TAC should decrease in LPS vs. control chicks, though we could not predict differences in oxidative response to LPS challenge between brood types. Finally, we investigated whether the effects of brood type and immune challenge on growth and physiology were sex-specific in a species where males are larger than females already at the chick stage (see "Results"; Chin et al. 2005). To our knowledge, this is one of the very few studies investigating variation in nestling quality (in terms of oxidative response to an immune challenge) between first and second broods in relation to sex.

Materials and methods

Field procedures and immune challenge

The study was carried out in a nestbox breeding population of starlings (74 nestboxes installed) near Ozzano Emilia (N Italy), during spring–summer 2010. The colony is located within a ca. 30-ha set-aside and naturalized area, surrounded by cornfields (>90% of the surface within a radius of 1 km); the neighbouring landscape also hosts several rural buildings with small orchards and a horse racecourse. Nestboxes were made of softwood panels (2 cm thick), with inside dimensions 15×15 cm (base)×45 cm (height) and entrance hole size diameter of 4.5 cm (distance of the hole from the base was 31 cm). Nestboxes were set up for the first time during early spring 2009. In 2010, occupancy rate (total number of nestboxes where at least one incubated clutch was laid over the entire season, i.e. including both first and second clutches) was 38%. In the study population, starlings

lay two clutches per season consisting of two to nine eggs (2009–2010, mean size of first clutch—5.1 (0.3 s.e.) eggs, n=26; second clutch—4.8 (0.1 s.e.) eggs, n=38). Mean laying dates of first and second clutches differ by more than 1 month and do not overlap (2009–2010; mean laying date, first clutches—13 April; second clutches—20 May). Among the clutches included in this experiment, first ones (n=8) were started between 6 and 19 April, while second ones (n=14) between 5 and 22 May. A few so-called intermediate clutches (n=3) (Pinxten et al. 1990) were excluded from the experiment. Only three nestboxes were occupied during both the first and the second brood, since most females likely changed nestbox between broods (see below; nestbox and mate changes between first and second broods occur frequently in starlings; Feare and Burham 1978). Nestboxes were not cleaned after fledging of first-brood chicks. As also reported in the literature (Cramp 1998), fledging success of second clutches (number of chicks fledged on clutch size) was lower than that of first clutches (2010, mean fledging success, first clucthes—0.60±0.10 s.e., n=12 clutches; second clutches—0.30±0.10 s.e., n=24 clutches). In the set of clutches included in the present experiment, all hatched chicks from first clutches successfully reached fledging age (19 days), whereas mortality occurred only among second-clutch chicks (see below and "Results"). Since most adult birds were not marked, we could not identify parental identity of many focal nestboxes. Therefore, we cannot exclude that some second clutches were very late first clutches, though this is unlikely given the high synchrony of both first and second clutches, well-spaced laying dates and exclusion of 'intermediate' clutches (see above). However, four females that were trapped and ringed at nestboxes while rearing first broods were retrapped while rearing the second clutch (one female in the same nestbox and the others in different nestboxes). Nestbox content was checked every 1–3 days during egg laying and every 1–2 days after hatching. At day 8 post-hatching, half of the chicks of each nest were subjected to an immune challenge with LPS, whereas the other half were subjected to a control treatment (in case of an odd number of nestlings, odd chicks were assigned at random to treatments). Fifty microlitres of a solution of 1 mg lyophilized LPS powder (026:B6 serotype, L8274, Sigma-Aldrich) diluted in 1 ml phosphate-buffered saline (PBS) was injected intraperitoneally. Since body mass of starling chicks at day 8 is ca. 45 g (46.47 g±1.10 s.e. in our sample of nestlings), the amount of LPS we chose to inject corresponds to ca. 1 mg/kg body mass, similarly to doses used in some previous studies (e.g. Alonso-Alvarez et al. 2004; Bertrand et al. 2006; Berthouly et al. 2008; Grindstaff 2008; Romano et al. 2011). Control nestlings were injected with the same amount of PBS only. Chick morphology [body mass, to the nearest 0.1 g with an electronic balance; tarsus length and length of the growing first primary feather (numbered descendantly; feather length hereafter) to the nearest 0.1 mm with dial calliper] was recorded on day 8 (before the immune challenge) and on day 9 (24 h after the immune challenge). In a subsample of birds, we also measured body mass at day 1 post-hatching (n=27 chicks from first broods and n=15 from second broods) that closely mirrors egg mass (Williams 1994; Krist 2011) and near fledging (17 days post-hatching; Chin et al. 2005; n=37 chicks from first broods and n=6 from second broods). We found that body mass at days 8–9 (mean value of measurements taken at both ages) strongly positively predicted body mass at days 16–17 (mean value of measurements taken at both ages) (first brood chicks: r=0.72, P<0.001, n=37; second brood chicks: r=0.81, P=0.054, n=6). Therefore, body mass at days 8–9 reliably reflects that at the end of the nestling period. On day 9, a blood sample (ca. 150 μl) was drawn from the brachial vein into microhematocrit capillary tubes and kept cool until processing (within a few hours, see below).

Sex determination and assay of plasma TAC and ROM concentration

Blood samples were centrifuged at 11,500 rpm for 10 min (centrifuge radius 94 mm) and plasma separated from red blood cells (RBC). Hematocrit (proportion of RBC over total blood volume) was measured on capillary tubes with a ruler (nearest 0.5 mm). Plasma and RBC were then stored at −80°C until analyses.

Molecular sexing was performed using the method originally developed by Griffiths et al. (1998). We amplified part of the W-linked avian CHD gene (CHD-W) in females and its non-W-linked homologue (CHD-Z) in both sexes using polymerase chain reaction (see Griffiths et al. 1998 for details of procedure). All nestlings subject to this procedure were successfully sexed.

The plasma antioxidant barrier includes both exogenous (e.g. ascorbate, tocopherols, carotenoids) and endogenous (e.g. uric acid, enzymes) compounds (Costantini 2008; Monaghan et al. 2009). Plasma TAC was measured using the OXY-Adsorbent test (Diacron, Grosseto, Italy). This test uses a colorimetric determination to quantify the ability of the plasma antioxidant barrier to cope with the oxidant action of hypochlorous acid (HClO). Briefly, plasma (5 μl) was diluted 1:100 with distilled water. A 5-μl aliquot of the diluted plasma was added to 200 μl of a titred HClO solution. The solution was gently mixed and incubated for 10 min at 37°C. At the end of the incubation time, 5 μl of an alkyl-substituted aromatic amine solubilized in a chromogenic mixture was added. Such amine is oxidized by the residual HClO and transformed in a pink-coloured derivative. The concentration of coloured complex is directly proportional to the HClO excess and inversely related to

the antioxidant capacity of tested plasma. The intensity of the coloured solution was measured at 492 nm using a photometer (Multiskan EX, Labsystem). One standard sample of known TAC and one blank sample (5 μl of distilled water) were processed and used as reference. Antioxidant capacity is expressed as millimolars of HClO neutralised.

ROM are early peroxidation products of the exposure of biological macromolecules (such as proteins, lipids and nucleic acids) to reactive oxygen species (ROS) (Costantini 2008; Monaghan et al. 2009). ROM are relatively more stable than ROS, and therefore, they can be conveniently detected and quantified (Costantini 2008; Monaghan et al. 2009). The plasma concentration of ROM (primarily hydroperoxides, ROOH) was measured by the d-ROMs test (Diacron, Grosseto, Italy). The plasma (10 μl) was diluted with 200 μl of a solution containing an acetate buffer (pH 4.8) and an alkyl-substituted aromatic amine solubilised in a chromogenic mixture. The solution was gently mixed and then incubated for 75 min at 37°C. During incubation, the acidic pH of the acetate buffer favoured the iron release from plasma proteins. This metal catalysed the cleavage of ROOH in two different free radicals. Such radicals are able to oxidize the alkyl-substituted aromatic amine solubilized in the chromogen producing a pink-coloured derivative whose colour intensity is directly proportional to the concentration of ROM. After incubation, the absorbance was read at 492 nm using a photometer (Multiskan EX, Labsystem). One standard sample and one blank sample (10 μl of distilled water) were processed and used as reference. The results of d-ROMs test are expressed as millimolars of H_2O_2 equivalents.

Repeatability of TAC and ROM measurements, as assessed by the intraclass correlation coefficient of 20 individuals that were assayed twice, was high and statistically significant in both cases (TAC: R=0.56, $F_{19,20}$=3.54, P=0.004; ROM: R=0.56, $F_{19,20}$=3.59, P=0.003). Intra- and inter-assay coefficients of variation were, respectively, as follows: TAC, 5.0% and 7.1%; ROM, 3.3% and 5.2%.

Statistical analyses

Variation in chick phenotypic traits in relation to brood (first vs. second), immune challenge and sex was investigated by means of mixed models. For traits measured both before and after immune challenge (body mass, tarsus and feather length), we included both nestbox and chick identity as nested random effects and included age (day 8 or 9 post-hatching) as an additional fixed factor. For traits measured only after the immune challenge (hematocrit, TAC, ROM), we included nestbox identity as a random factor. Interactions (up to the highest level of complexity) were included in initial full models. Final models were obtained by removing from the full model in a single-step all non-significant interactions of a given order. However, if a statistically significant interaction emerged, all interactions of the same order (and those of inferior orders) were kept in the final model. With this procedure, we aimed at reducing the probability of committing type I errors due to multiple statistical tests, as occurs with traditional stepwise procedures (e.g. Whittingham et al. 2006). The above analyses were repeated for the subset of chicks that could be attributed to the same four mothers (see above) during each clutch, though with following differences in model specifications: (1) four-way interactions were not tested in models of morphological traits because sample size was too small, and (2) female identity was included as an additional random effect in all models.

The analysis of survival to fledging was conducted on second-brood chicks only (all first-brood chicks fledged successfully) by means of a binomial mixed model (dependent variable coded as survivor=1 and non-survivor=0) with nestbox identity as a random grouping factor and immune challenge, sex and hatch date as predictors. Interactions could not be tested in this model because the design was poorly saturated (among non-surviving chicks, all but one were females; see "Results") and complex models did not converge (details not shown for brevity). The model was not overdispersed, and no correction to standard errors was therefore applied (Zuur et al. 2009). Finally, we compared the phenotype of second-brood females (no first-brood chick died and only a single second-brood male died; see above and "Results") that survived vs. those that died before fledging with mixed models, where female phenotypic traits were included as dependent variables and survival to fledging as a fixed factor. In the models of female body mass, tarsus and feather length, we included nestbox and chick identity as nested random effects (to account for repeated measurement of the same chick in consecutive days, days 8 and 9 post-hatching), whereas in models of hematocrit, TAC and ROM, we included only nestbox identity as a random effect. All analyses were carried out with the MIXED and GLIMMIX procedures of SAS 9.1.3 (Littell et al. 2006). In Gaussian mixed models, degrees of freedom were estimated by the Kenward–Rogers method, which provides a conservative estimate of the denominator degrees of freedom (Littell et al. 2006). Overall, analyses were carried out on 41 chicks [19 males (9 control; 10 LPS-injected) and 22 females (10 controls; 12 LPS-injected)] from 8 first broods and 45 chicks [15 males (8 controls, 7 LPS-injected) and 30 females (13 controls, 17 LPS-injected)] from 14 second broods. The analyses carried out on the subset of chicks from the same mothers were based on 36 chicks (21 from first broods and 15 from second broods). Sample sizes may vary slightly between analyses because of missing data (due accidental reasons, such as blood sample loss or amount too small for biochemical analyses).

Information on sample sizes is also reported throughout the "Results" and in figure captions.

Results

Variation in oxidative status and hematocrit

The analyses based on the complete dataset showed that plasma TAC and ROM concentration were unaffected by immune challenge (Table 1), but TAC of second-brood chicks was significantly lower than that of first-brood ones (Table 1; Fig. 1). Hematocrit was significantly lower among second- than first-brood chicks (Table 1; Fig. 1). Moreover, hematocrit was affected by immune challenge among second- but not first-brood chicks, with second-brood LPS-chicks showing a significantly higher hematocrit than controls (Table 1; Fig. 1). All these findings were qualitatively unaltered when the analyses were repeated on the subset of chicks reared by the same mothers in both the first and second brood (Table 1).

Variation in growth

Based on the complete dataset, models showed that, at days 8–9 post hatching, second-brood chicks were ca. 19% lighter than first-brood ones (Table 2; Fig. 2). Tarsus length was also significantly smaller among second-brood chicks, whereas feather length did not differ (Table 2; Fig. 3). Increase of body mass between day 8 and day 9 post-hatching differed between sexes in a brood order-specific way, as testified by the statistically significant three-way interaction between brood type, sex and age (Table 2): post hoc comparisons revealed that among first-brood chicks, males were non-significantly larger than females at both ages (day 8, t=0.37, P=0.71; day 9, t=1.85, P=0.068), and body mass of both sexes increased significantly between day 8 and day 9 (males, t=8.63, P<0.001; females, t=4.80, P<0.001), while among second-brood chicks males were markedly heavier than females at both ages (both t>4.08, P<0.001), but body mass did not increase significantly from day 8 to day 9 (males, t=0.89, P=0.38; females, t=1.12, P=0.27) (Fig. 2).

Table 1 Mixed models of TAC, ROM and hematocrit of nestling starlings at day 9 (i.e. 24 h after the immune challenge) based on the entire dataset (n=86) or on the subset of chicks raised by the same mothers in both the first and the second brood (n=36)

Variable	All chicks[a]			Same mothers[b]		
	F	*df*	*P*	*F*	*df*	*P*
TAC						
Brood	22.28	1, 57.7	< 0.001	15.37	1, 9.1	0.003
Immune challenge	1.72	1, 59.3	0.19	2.40	1, 27.0	0.13
Sex	0.28	1, 68.0	0.60	0.09	1, 29.8	0.77
Dropped terms						
Brood × immune challenge	0.03	1, 56.9	0.86	0.19	1, 23.9	0.67
Brood × sex	0.01	1, 63.7	0.96	0.28	1, 27.2	0.60
Immune challenge × sex	0.11	1, 64.7	0.74	0.34	1, 26.2	0.56
Brood × immune challenge × sex	0.19	1, 64.6	0.66	0.26	1, 24.3	0.62
ROM						
Brood	0.03	1, 48.5	0.86	0.05	1, 17.7	0.82
Immune challenge	0.36	1, 61.3	0.55	0.05	1, 25.0	0.82
Sex	0.02	1, 69.9	0.88	0.08	1, 28.1	0.78
Dropped terms						
Brood × immune challenge	2.20	1, 58.9	0.14	1.91	1, 21.4	0.18
Brood × sex	2.60	1, 64.7	0.11	0.82	1, 24.1	0.37
Immune challenge × sex	1.40	1, 65.5	0.24	3.89	1, 22.4	0.06
Brood × immune challenge × sex	0.49	1, 65.0	0.49	0.36	1, 21.6	0.56
Hematocrit						
Brood	14.28	1, 66.0	0.003	15.37	1, 8.2	0.004
Immune challenge	6.01	1, 50.9	0.018	6.38	1, 23.7	0.019
Sex	3.26	1, 60.5	0.08	3.13	1, 26.4	0.09
Brood × immune challenge	8.60	1, 51.8	0.005	7.70	1, 23.8	0.010
Brood × sex	1.66	1, 57.3	0.20	0.52	1, 26.4	0.48
Immune challenge × sex	0.08	1, 58.1	0.78	0.14	1, 26.0	0.71
Dropped terms						
Brood × immune challenge × sex	2.79	1, 59.0	0.10	0.04	1, 23.6	0.84

See "Materials and methods" for details on model simplification procedures. Degrees of freedom were estimated by the Kenward–Rogers method

[a]Mixed models with nestbox identity as a random factor

[b]Mixed models with nestbox and mother identity as random factors

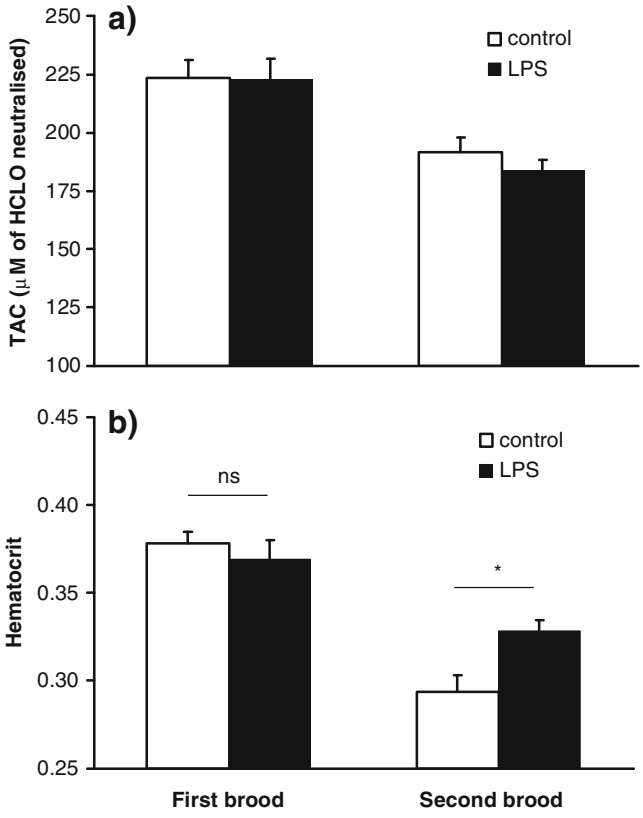

Fig. 1 Mean (+ s.e.) values of total antioxidant capacity (*TAC*) and hematocrit (proportion of red blood cells) of first- and second-brood nestling starlings in relation to LPS challenge. Sample size is 40 chicks from first broods (19 controls and 21 LPS-injected) and 42 from second broods (19 controls and 23 LPS-injected); *ns* not significant ($P>0.05$); *$P<0.001$ at post hoc tests

Tarsus length of first-brood chicks was larger than that of second-brood ones (Table 2; Fig. 3). Female tarsi were smaller than male ones and especially so among chicks from second broods (Table 2; Fig. 3). Similarly, males had longer feathers than females among second- but not first-brood chicks (Table 2; Fig. 3). Furthermore, growth of wing feathers between day 8 and day 9 was faster among first- than among second-brood chicks (Table 2; Fig. 3). Immune challenge did not affect body mass, tarsus or feather length among either first- or second-brood chicks (Table 2).

The analyses carried out on the reduced set of chicks reared by the same mothers during the first and second brood were broadly supportive of the above findings (Table S1). For body mass, the three-way interaction between brood type, sex and age was only marginally non-significant ($F_{1,29.0}=3.18$, $P=0.085$), notwithstanding the much smaller sample size. Brood-specific sex dimorphism in body mass was confirmed (brood×sex interaction: $F_{1,26.2}=4.33$, $P=0.047$; Table S1). However, in the reduced dataset, tarsus length did not differ between first- and second-brood chicks, and there was no differential effect of brood type on sex dimorphism in tarsus length (Table S1). Feather growth was faster among first- vs. second-brood chicks, although at both ages wing feathers of second-brood chicks were significantly longer

Table 2 Mixed models (with nestbox and chick identity as random factors) of body mass, tarsus length and feather length variation between day 8 and day 9 post-hatching in nestling starlings based on the entire dataset

Variable	*F*	*df*	*P*
Body mass			
Brood	16.77	1, 62.1	<0.001
Immune challenge	0.54	1, 58.2	0.46
Sex	14.41	1, 67.6	<0.001
Age	58.76	1, 74.8	<0.001
Brood×immune challenge	0.23	1, 58.4	0.64
Brood×sex	5.15	1, 65.4	0.027
Brood×age	32.28	1, 74.8	<0.001
Immune challenge×sex	0.38	1, 64.9	0.54
Immune challenge×age	2.95	1, 74.8	0.09
Sex×age	4.58	1, 74.8	0.036
Brood×immune challenge×sex	0.46	1, 64.9	0.50
Brood×immune challenge×age	2.46	1, 74.9	0.12
Brood×sex×age	4.38	1, 74.8	0.039
Immune challenge×sex×age	0.68	1, 74.8	0.61
Tarsus length			
Brood	10.13	1, 68.0	0.002
Immune challenge	1.17	1, 58.1	0.28
Sex	10.80	1, 66.4	0.002
Age	111.53	1, 77.2	<0.001
Brood×immune challenge	1.17	1, 58.5	0.28
Brood×sex	4.60	1, 64.2	0.036
Brood×age	0.03	1, 77.2	0.86
Immune challenge×sex	0.05	1, 63.6	0.82
Immune challenge×age	1.13	1, 77.3	0.29
Sex×age	0.18	1, 77.1	0.67
Feather length			
Brood	0.03	1, 78.2	0.86
Immune challenge	0.09	1, 60.2	0.77
Sex	1.07	1, 64.8	0.30
Age	263.77	1, 77.5	<0.001
Brood×immune challenge	0.02	1, 60.4	0.86
Brood×sex	3.87	1, 63.2	0.053
Brood×age	75.48	1, 77.5	<0.001
Immune challenge×sex	0.17	1, 62.8	0.68
Immune challenge×age	0.81	1, 77.6	0.37
Sex×age	0.05	1, 77.4	0.83

Four-way interactions were not significant in any case and were removed from the models (all $P>0.49$). Three-way interactions were also not significant for models of tarsus and feather length (all $P>0.24$; see "Materials and methods" for details on model simplification procedures). Degrees of freedom were estimated by the Kenward–Rogers method

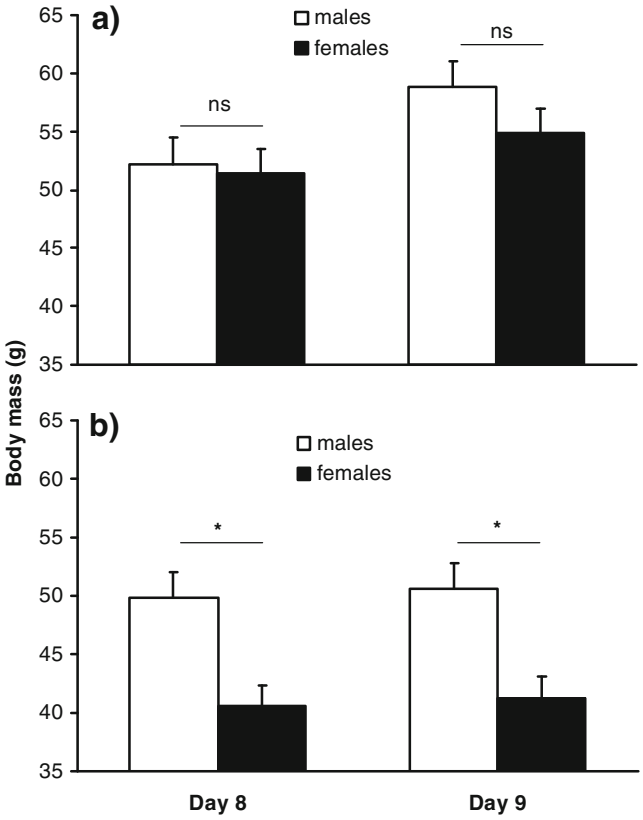

Fig. 2 Mean (+ s.e.) values of body mass of **a** first- and **b** second-brood nestling starlings in relation to age and sex (values represent model-estimated means from the model shown in Table 2). Sample size is 41 chicks from first broods (19 males and 22 females) and 45 from second broods (15 males and 30 females); ns: not significant ($P>0.05$); *$P<0.001$ at post hoc tests

than those of first-brood ones (post hoc tests, $P<0.04$) (see main effect of brood in Table S1). Brood-specific sex dimorphism in feather length was not confirmed (Table S1), but this effect was weak also in the model based on the entire dataset (see Table 2). Minor discrepancies with respect to the results based on the entire dataset may reflect sampling effects due to the small sample size and will thus not be discussed further.

Survival to fledging of second-brood chicks in relation to sex, immune challenge and phenotype

This analysis was performed only for second-brood chicks because all 41 chicks from first broods fledged successfully. On the other hand, 14 out of 45 (31%) chicks from second broods died before fledging (mean age of death was 12 days post-hatching, range 7–19). Among chicks that died before fledging, all but one were females. Thus, in a binomial mixed model with treatment, sex and hatching date as predictors and nestbox identity as a random factor, probability of surviving to fledging was predicted by chick sex, with males surviving better than females ($F_{1,28}=4.23$, $P=0.049$), whereas the effects of immune challenge and hatch date were non-significant ($F_{1,28}=0.01$, $P=0.91$ and $F_{1,28}=2.27$, $P=0.14$, respectively; interactions were not tested, see "Statistical analyses" for details). Body mass and tarsus length were significantly larger among surviving female chicks compared to non-surviving ones, whereas all other traits did not significantly differ between surviving and non-surviving females (Table 3).

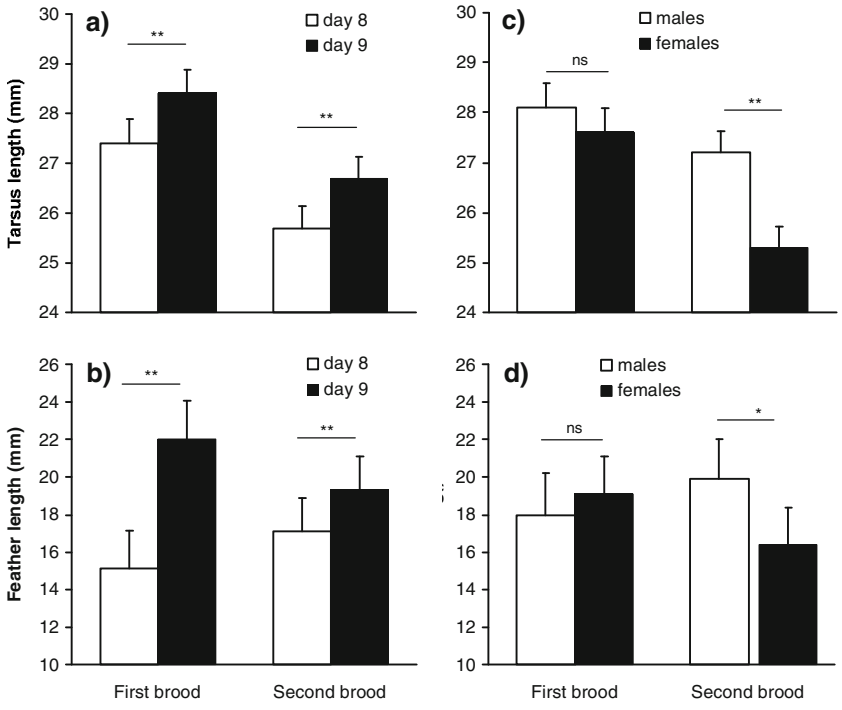

Fig. 3 Mean (+ s.e.) values of tarsus and feather length of first- and second-brood nestling starlings in relation to age (*left column*, **a** and **b**) and sex (*right column*, **c** and **d**) (values represent model-estimated means from models listed in Table 2). Sample size is 41 chicks from first broods (19 males and 22 females) and 45 from second broods (15 males and 30 females); *ns* not significant ($P>0.05$); *$P<0.045$; **$P<0.001$ at post hoc tests

Table 3 Phenotype (mean and s.e.) of second-brood females surviving (n=17) or not (n=11) to fledging

Trait	Surviving	Non-surviving	F	df	P
TAC	181.90 (10.33)	193.65 (12.59)	0.56	1, 21.3	0.46
ROM	1.77 (0.17)	1.50 (0.23)	0.90	1, 15.2	0.36
Hematocrit	0.30 (0.01)	0.31 (0.02)	0.10	1, 22.4	0.75
Body mass (g)	45.13 (2.76)	36.91 (2.80)	6.99	1, 25.8	0.014
Tarsus length (mm)	26.49 (0.78)	24.40 (0.81)	4.92	1, 27.6	0.035
Feather length (mm)	19.50 (2.82)	14.09 (2.85)	3.86	1, 23.2	0.062

Values represent model-estimated parameters from mixed models with nestbox identity as a random factor (TAC, ROM, hematocrit) or mixed models with nestbox and chick identity as random factors (body mass, tarsus length, feather length; all measurements taken at days 8 and 9 post-hatching). Data from two females that died before day 8 were not available. Degrees of freedom were estimated by the Kenward–Rogers method

Discussion

In this study, we examined seasonal variation in offspring quality by subjecting first- and second-brood starling chicks to an immune challenge with LPS and evaluated their growth and physiological response in terms of plasma TAC, ROM and hematocrit. LPS challenge did not affect growth or physiological condition of both first- and second-brood chicks of either sex, with the single exception of hematocrit, that was higher among second-brood (but not among first-brood) LPS chicks compared to controls. As expected, we found that second-brood chicks were in poorer condition than first-brood ones, and such differences were sex-specific for body mass at days 8–9 post-hatching. Importantly, the most relevant findings were qualitatively unaltered when the analyses were restricted to the subset of chicks reared by the same mothers during both the first and the second brood, strongly suggesting that any brood-specific pattern we detected on the entire population did not originate from genetic differences between parents occupying experimental nestboxes early and late in the season.

Seasonal decline in condition and antioxidant defences

Chicks from first and second clutches showed remarkable differences in most of the condition indices we measured. Halfway through the rearing period, chicks from first broods were heavier than those of second broods and grew at a faster rate. Moreover, hematocrit and TAC were significantly lower among second-brood chicks. These findings indicate that the nutritional conditions of starling chicks worsened in the course of the season between first and second clutches (see "Introduction"; López-Rull et al. 2011) and that this may impair functioning of the antioxidant barrier (Monaghan et al. 2009).

Although the use of hematocrit as a condition index is widely debated (Fair et al. 2007), our finding that hematocrit of second-brood chicks was smaller than that of first-brood ones may suggest that the former were in poor nutritional state, had higher parasite burden or both, as shown by some previous studies of wild nestling birds (e.g. Richner et al. 1993; Merino and Potti 1998; Potti et al. 1999; Simon et al. 2005). The decline of TAC between first and second broods we observed corroborates recent reports by Costantini et al. (2010) and Norte et al. (2009) of a seasonal decline in the capacity to resist oxidative stress among nestling birds. Antioxidant defences have an important environmental component (Costantini and Dell'Omo 2006a; Rubolini et al. 2006; Norte et al. 2009) and may thus reliably reflect ecological conditions to which nestling birds are exposed to. Indeed, the relevance of the rearing environment in determining chick oxidative status was recently highlighted by a study of starlings showing that TAC was lower among experimentally enlarged broods compared to reduced ones, but only in a poor year in terms of ecological conditions (Bourgeon et al. 2011). In addition, ROM levels, though not affected by the harshness of within-brood competition, were 45% higher in a poor vs. a good year (Bourgeon et al. 2011).

Several mechanisms may concur to originate a reduced antioxidant capacity of second-brood chicks: for example, it may be a direct consequence of seasonal changes of antioxidant availability in nestling diet (Catoni et al. 2008), it may result from seasonal changes in maternal effects via egg quality mediated by a decline in parental phenotype (Rubolini et al. 2006; López-Rull et al. 2010), or from the observed nutritional deficiencies of second-brood nestlings (as indexed by their lower body mass) (see also Monaghan et al. 2009) due to seasonally deteriorating ecological conditions. In addition, a lower TAC among second-brood chicks may depend on a higher parasite load compared to first-brood ones (López-Rull et al. 2010).

Brood- and sex-specific growth patterns and mortality

Male chicks were larger than female ones, but among second-brood chicks the extent of sexual dimorphism was more pronounced compared to first-brood ones. Halfway through the rearing period, males were 4.7% heavier than

females in first-brood chicks, but 22.7% heavier in second-brood ones. Thus, under poorer rearing conditions, size dimorphism in favour of the larger sex was increased (see Oddie 2000 for a similar finding in *Parus major*). This pattern may originate from seasonal and sex-specific maternal investment in egg mass. Indeed, an analysis of body mass at day 1 of age (that closely mirrors egg mass; see Krist 2011) of a subsample of 42 chicks (see "Materials and methods") revealed that body mass at hatching varied according to the combined effects of sex and brood (mixed model with nestbox identity as a random factor, $F_{1,28.5}$= 8.97, P=0.006), males being smaller than females among first-brood chicks (post hoc test, t=−2.24, P=0.034) whereas the opposite was the case among second-brood chicks (t=2.11, P=0.045). Thus, between day 1 and day 9, the size advantage of first-brood females at hatching weakened and males became larger, whereas males remained larger than females between day 1 and day 9 among second-brood chicks. An ontogenetic shift of sexual size dimorphism among first-brood chicks is in line with previous findings documenting larger female vs. male eggs in first clutches of the closely related *Sturnus unicolor* (Cordero et al. 2001).

Among altricial offspring, body size during the pre-fledging period, mostly mirroring hatch order, is an important determinant of the success in scrambling competition for access to food (e.g. Price and Ydenberg 1995; Slagsvold et al. 1995; Cotton et al. 1999; Oddie 2000). Thus, starling mothers may provide daughters with an early size advantage in first clutches in order to promote their competitiveness against larger sons and thus enhance their fitness prospects. Indeed, among first clutches, where females were larger than males at hatching, size dimorphism halfway through the rearing period was far less pronounced than among second-brood chicks. Moreover, pre-fledging mortality of second-brood chicks was strongly female-biased, and body size of females that survived to fledging age was larger than those not surviving, suggesting that a body size advantage may have significant fitness consequences even during the pre-fledging stage in this species. A sex-biased mortality during the pre-fledging stage has been repeatedly shown in several bird species (see reviews in Råberg et al. 2005 and Uller 2006), but to our knowledge, a difference in sex-specific survival to fledging between first and second broods has never been previously reported.

In starlings, a larger investment in female vs. male offspring early but not late in the season may be adaptive since probability of recruitment and breeding of females during their first breeding season after hatching may depend on fledging date, early fledging females having a higher probability of breeding when 1 year old, as shown in a study of *S. unicolor* by Cordero et al. (2001). On the other hand, for males *S. unicolor* that do not breed during their first breeding season after hatching, the probability of recruitment to the breeding population was only positively related to their body mass at hatching (Cordero et al. 2001). Our findings are therefore consistent with maternal effects via egg mass or composition favouring daughters early in the season, but males later on (Cordero et al. 2001).

The causes of female-biased mortality among poor quality second-brood chicks require further scrutiny. Individuals of the larger sex (usually males) are regarded as being more susceptible than those of the smaller sex (usually females) to harsh environmental circumstances because of their greater energetic requirements during growth (Clutton-Brock et al. 1985). On the other hand, asymmetries in competitive abilities due to sex differences in body size may counterbalance and even outweigh male energetic penalties (Uller 2006). The balance between these two opposing forces determining offspring fitness may be resolved by parental decisions that can favour either smaller or larger chicks depending on fitness payoffs. In the first case, which is typical of many passerine species with limited hatching asynchrony, parents tend to equalize competitive asymmetries by adopting a so-called brood survival strategy (Slagsvold et al. 1984), whereby parents reduce competitive gaps by preferentially feeding smaller, less competitive chicks that beg more vigorously (Bonisoli-Alquati et al. 2011). On the other hand, under unfavourable environmental circumstances, parents may reduce provisioning of small, poor quality chicks of low reproductive value and invest more into high quality chicks that may have higher chances of surviving to maturity, a strategy that may lead to brood reduction (Clark and Wilson 1981; Slagsvold et al. 1984). The latter is what seems to happen with second-brood females that are likely to be of low reproductive value because they are smaller than males at hatching and throughout rearing, and suffer high pre-fledging mortality. It would be interesting to disentangle whether female-biased mortality of second-brood chicks occurred via parental discrimination favouring male offspring or intense sibling competition favouring the larger males (e.g. Cotton et al. 1999).

Effects of LPS challenge on offspring phenotype

LPS challenge had no detectable effects on offspring growth and physiology of first-brood chicks, though it affected hematocrit of second-brood chicks. The dose injected was similar to that used in previous studies where it was shown to cause a rapid negative effect on chick growth (Grindstaff 2008; Romano et al. 2011) (but see Berthouly et al. 2008). These studies were, however, based on a larger sample size as compared to ours, and a reduced power in our study may contribute to explaining non-significant results. Although we cannot exclude that negative effects on body mass emerged after day 9, this seems unlikely since LPS did not affect chick body mass a few days before fledging (days 16–

17) in the subsample of birds that was remeasured at that age (details not shown). Thus, assuming that the lack of detectable effects of LPS on chick traits is not a mere consequence of the relatively small sample size (though sample size was much larger than that of previous studies investigating the oxidative costs of immune response, see Costantini and Møller 2009), European starlings may be able to sustain the challenge imposed to the immune system by a bacterial endotoxin by paying a relatively small energetic cost (either direct or indirect, see "Introduction"), at least until fledging, even among nutritionally stressed second-brood chicks. We might only tentatively speculate about the possible causes of such an apparently minor effect of LPS challenge on starling chick fitness. For example, starlings are among the bird species showing the highest prevalence of *E. coli* (Morishita et al. 1999), and transgenerational priming of the offspring immune system by transmission of maternal antibodies towards *E. coli* via the eggs might at least partly buffer the costs of mounting an immune response, as shown by Grindstaff et al. (2006) and Grindstaff (2008) in both wild pied flycatchers (*Ficedula hypoleuca*) and captive Japanese quails (*Coturnix japonica*), respectively. In addition, the European starling is a colonial and cavity-nesting species, both conditions leading to a larger size of primary immune defence organs according to comparative evidence (Møller and Erritzøe 1996) and might have thus evolved a highly efficient system of defence against pathogen exposure (Møller and Erritzøe 1996; Møller et al. 2009). A previous study also suggested that the evolutionary and ecological history of a population, such as intense past selection for resistance to bacterial attacks in the present case, could play a role in the apparent lack of short-term response to LPS challenge (see, e.g. Lee et al. 2005 for lack of response in *Passer domesticus* vs. strong response in *Passer montanus*).

The increased hematocrit among second-brood LPS chicks is difficult to interpret (Fair et al. 2007) but may be a consequence of the rapid metabolic changes induced by LPS challenge (e.g. dehydration following a febrile state or variation in metabolic rate). A raise in hematocrit may thus be a sentinel of the higher maintenance costs of responding to LPS challenge among poor-condition second-brood chicks, whose hematocrit was also significantly lower than that of first-brood ones. This is in line with the prediction that the effect of immune challenge was stronger among poor-condition second-brood chicks compared to prime condition first-brood ones.

Some previous studies, including a meta-analysis, showed that immune challenge may affect oxidative status in avian species (Bertrand et al. 2006; Costantini and Dell'Omo 2006b; Costantini and Møller 2009). However, the meta-analysis highlighted significant heterogeneity in effect sizes, likely resulting from population-, dose- or antigen-specific differences (Costantini and Møller 2009) (see, e.g. Alonso-Alvarez et al. 2004; Horak et al. 2006 for studies not showing significant effects). Alternatively, a lack of response may result from compensatory (up)regulation of the antioxidant system, whose costs may be paid up later in life. However, the lack of effect of immune challenge on oxidative status markers is consistent with observed lack of effect on body growth, further corroborating the idea that immune challenge with the dose and LPS serotype we injected may not impose detectable costs to nestling starlings, at least in the short-term.

In conclusion, our study revealed a generalized seasonal decline in fitness-related traits of starling chicks, whose consequences were more severe for the smaller female offspring, suffering higher prefledging mortality than males in second broods. This larger seasonal decline of daughters' fitness may be mediated by seasonal variation in sex allocation by mothers. Furthermore, our study indicates that nestling starlings may be able to sustain an immune challenge, even if not in prime conditions, by paying a relatively small cost, possibly because they have evolved a highly efficient response to pathogen attacks. Finally, since antioxidant capacity is known to predict long-term survival (Bize et al. 2008; Saino et al. 2011), we suggest that a seasonal decline of antioxidant activity should be regarded among the proximate mechanisms generating a decline of long-term survival and recruitment prospects among late-season nestlings of bird species.

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Ethical standards This research was undertaken (capture and experimental treatments) under the combined prescriptions of Art. 4 (1) and Art. 7 (5) of the Italian law 157/1992, which regulates studies on wild bird species.

Conflicts of interest The authors declare that they have no conflict of interest.

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ELECTRONIC SUPPLEMENTARY MATERIAL

Table S1. Mixed models (with nestbox, mother and chick identity as random factors) of body mass, tarsus and feather length variation between day 8 and day 9 post-hatching in nestling starlings based on the subset of chicks that were reared by the same mothers during each brood. Four-way interactions were not tested (see Methods for details of model specification). To facilitate comparisons, we show the models with the same structure as those presented in Table 2. Three-way interactions in models of tarsus and feather length were invariably far from significance (all $P > 0.37$). Degrees of freedom were estimated by the Kenward-Rogers method.

Variable	*F*	*df*	*P*
Body mass			
Brood	8.88	1, 25.1	0.006
Immune challenge	0.46	1, 25.2	0.50
Sex	21.22	1, 25.6	< 0.001
Age	23.47	1, 29.0	< 0.001
Brood × Immune challenge	0.35	1, 25.1	0.56
Brood × Sex	4.33	1, 26.2	0.047
Brood × Age	14.85	1, 29.0	< 0.001
Immune challenge × Sex	2.24	1, 25.2	0.15
Immune challenge × Age	0.76	1, 29.0	0.39
Sex × Age	1.53	1, 29.0	0.23
Brood × Immune challenge × Sex	3.10	1, 26.1	0.09
Brood × Immune challenge × Age	0.97	1, 29.0	0.33
Brood × Sex × Age	3.18	1, 29.0	0.08
Immune challenge × Sex × Age	0.24	1, 29.0	0.63
Tarsus length			
Brood	1.58	1, 5.7	0.26
Immune challenge	0.15	1, 23.8	0.70
Sex	8.13	1, 26.2	0.008
Age	38.20	1, 32.0	< 0.001
Brood × Immune challenge	0.75	1, 23.9	0.39
Brood × Sex	2.22	1, 28.0	0.15
Brood × Age	0.05	1, 32.0	0.82
Immune challenge × Sex	1.70	1, 25.6	0.20
Immune challenge × Age	1.16	1, 32.0	0.29
Sex × Age	0.11	1, 32.0	0.75
Feather length			
Brood	9.14	1, 29.0	0.005
Immune challenge	0.40	1, 22.4	0.54
Sex	1.67	1, 24.4	0.21
Age	73.32	1, 32.0	< 0.001
Brood × Immune challenge	0.05	1, 22.3	0.82
Brood × Sex	0.59	1, 24.2	0.45
Brood × Age	23.71	1, 32.0	< 0.001
Immune challenge × Sex	0.46	1, 23.1	0.51
Immune challenge × Age	1.54	1, 32.0	0.22
Sex × Age	0.69	1, 32.0	0.41

PART III

LONG-TERM EFFECTS OF REARING CONDITIONS ON REPRODUCTIVE SUCCESS

Chapter 6

Longevity and lifetime reproductive success of barn swallow offspring are predicted by their hatching date and phenotypic quality

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Longevity and lifetime reproductive success of barn swallow offspring are predicted by their hatching date and phenotypic quality

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Summary

1. Longevity is a major determinant of individual differences in Darwinian fitness. Several studies have analyzed the stochastic, time-dependent causes of variation in longevity, but little information exists from free-ranging animal populations on the effects that environmental conditions and phenotype early in ontogeny have on duration of life.

2. In this long-term (1993–2011) study of a migratory, colonial, passerine bird, the barn swallow (*Hirundo rustica*), we analyzed longevity and, in a subsample of individuals, lifetime reproductive success (LRS) of the offspring that reached sexual maturity in relation to hatching date, which can affect the rearing environment through a seasonal deterioration in ecological conditions. Moreover, we analyzed the consequences of variation in body size and, for the first time in any species, of a major component of immunity on longevity, both by looking at absolute phenotypic values and at deviations from the brood mean.

3. Accelerated failure time models showed that individuals of both sexes that hatched early in any breeding season enjoyed larger longevity and larger LRS, indicating directional selection for early breeding. Both male and female offspring with large T cell-mediated immune response relative to their siblings and female nestlings that dominated the brood size/age hierarchy had larger longevity than their siblings of inferior phenotypic quality/age. Conversely, absolute phenotypic values did not predict longevity.

4. Frailty modelling disclosed marked spatial heterogeneity in longevity among colonies of origin, again stressing the impact of rearing conditions on longevity.

5. This study therefore reinforces the notion that perinatal environment and maternal decisions over timing and site of breeding, and position in the brood hierarchy can have marked effects on progeny life history that extend well into adulthood. In addition, it provides the first evidence from any bird population in the wild that immune response when nestlings predicts individuals' longevity after sexual maturation.

Key-words: hatching date, *Hirundo rustica*, immunity, life history, longevity, maternal effects

Introduction

The causes and mechanisms that generate variation in the relative contribution of individuals to the genetic composition of the next generations are pivotal to any research field in evolutionary ecology (Williams 1992). While genetic and environmental determinants of variation in Darwinian fitness have been often investigated using cross-sectional information on fecundity or progeny quality and short-term survival as proxies for reproductive success (e.g. Newton 1989), studies of offspring longevity in the wild are rare (Cam, Monnat & Hines 2003; Van de Pol *et al.* 2006; Wilkin & Sheldon 2009; Bouwhuis *et al.* 2010a,b). This is unfortunate because longevity is one of the key factors underpinning lifetime reproductive success (LRS) via an effect on number of reproductive events over the entire life (Newton 1989; Reid *et al.* 2003; Grant & Grant 2011).

Longevity has obvious environmental determinants, such as time-dependent exposure to predation or adverse weather

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(e.g. Newton 1998). However, environmental factors, including maternal effects (Mousseau & Fox 1998; Badyaev & Uller 2009; Wolf & Wade 2009), which offspring experience during early ontogeny, have the potential to act as major sources of variation in longevity but have been largely neglected in studies of wild populations (but see Cam, Monnat & Hines 2003; Van de Pol *et al.* 2006; Wilkin & Sheldon 2009; Bouwhuis *et al.* 2010b). The pathways whereby perinatal environment and maternal effects can translate into variation in offspring viability range from early epigenetic actions on germ cells to developmental consequences of variation in egg composition (Mousseau & Fox 1998; Eising *et al.* 2001; Saino *et al.* 2003, 2006; Rubolini *et al.* 2006; Muller *et al.* 2007; Badyaev & Uller 2009; Wolf & Wade 2009). Parental decisions on breeding time and site are also expected to set the early environmental scene for the offspring by affecting parental care and rearing conditions (Lindström 1999). Independent of the mechanism through which they operate, such effects can have immediate consequences on progeny viability but also on prime development, physiology and behaviour of the offspring and their life history, with consequences on performance later in life (Mousseau & Fox 1998; Alonso-Alvarez *et al.* 2006; Groothuis *et al.* 2006; Rubolini *et al.* 2006; Carere & Baltazhart 2007; Muller *et al.* 2007; Badyaev & Uller 2009; Schlichting & Mousseau 2009; Bonisoli-Alquati *et al.* 2011).

Timing of reproduction in seasonally varying environments typifies the effects of parental decisions over breeding on offspring. Seasonal deterioration of offspring quality and viability has been demonstrated in a number of studies, particularly of birds, and often interpreted as a consequence of the effects of worsening ecological conditions as the breeding season progresses on both parents and offspring (Lack 1950; Verboven & Visser 1998; Dubiec & Cichoñ 2001; Grüebler & Naef-Daenzer 2008; Verhulst & Nilsson 2008). Variation in phenotypic quality or age of parents breeding at different times constitutes an additional cause of variation in offspring phenotype (Newton 1989). Much work on early environmental and maternal effects in birds has used body size, immunity or recruitment into the breeding population as proxies for offspring fitness (Verboven & Visser 1998; Naef-Daenzer, Widmer & Nuber 2001; Verhulst & Nilsson 2008; López-Rull *et al.* 2011), but we are aware of extremely few studies where the effects of breeding date and phenotypic quality of the offspring on longevity *after sexual maturity* have been analyzed in any free-living bird population (e.g. Bouwhuis *et al.* 2010b), and even information from laboratory studies is scant (e.g. De Kogel 1997).

The first aim of our study thus was to analyze variation in longevity and LRS of barn swallows (*Hirundo rustica*) in relation to their hatching date. Specifically, we focused on longevity of sexually mature individuals recruited into the breeding population as 1-year old adults, because extremely high breeding philopatry allows to gather accurate information on annual survival. Conditions for breeding as well as parental quality in this species may deteriorate as the season progresses (Møller 1994; Turner 2006), and we therefore expected longevity after sexual maturity to decline with hatching date.

We also predicted that high phenotypic quality of individual offspring, as gauged by *absolute* body size, and, for the first time in any bird species, by a major component of immune response, predicts longevity. In altricial birds, however, hatching asynchrony often enforces an age/size hierarchy among siblings, which may result in severe competitive asymmetries: later hatched smaller offspring typically suffer restricted access to limiting parental resources compared to their siblings as a result of parental favouritism and/or differences in competitive abilities (Mock & Parker 1997). In such 'structured families' (sensu Hall *et al.* 2010), 'core', advantaged offspring enjoy better phenotypic conditions and viability (Lack 1954; Ricklefs 1993; Stenning 1996). In barn swallow broods, even small levels of hatching asynchrony (Boncoraglio & Saino 2008) translate into a size hierarchy which is permanently retained throughout the nestling period. Position in the brood hierarchy and social stress, however, may have long-lasting effects on longevity. We therefore also tested whether phenotypic quality of nestlings *relative* to their siblings predicted longevity while expecting a positive association between relative phenotypic value and longevity. Because nestlings in large broods experience poor nutritional conditions and harbour more ectoparasites per capita (Saino, Calza & Møller 1997; Saino *et al.* 2002a), we also had the prediction that longevity declined with brood size.

Sex is a major determinant of variation in longevity (Liker & Székely 2005), and sex-related variation in lifespan may depend on differential susceptibility to rearing conditions (Tschirren, Fitze & Richner 2003; Saino *et al.* 2006; Boncoraglio, Martinelli & Saino 2008). In the barn swallow, nestling sex ratio is close to parity (Saino, Martinelli & Romano 2008), while sex ratio among adults is male-biased (Møller 1994). We therefore investigated whether sex-related variation in longevity after sexual maturation existed, which may contribute to sex ratio variation among age classes.

Materials and methods

STUDY ORGANISM

The barn swallow is a small, semicolonial, socially monogamous passerine bird whose European breeding populations overwinter south of the Sahara (Møller 1994; Cramp 1998). The large majority of barn swallows breed colonially in rural buildings (Møller 1994; Ambrosini *et al.* 2002; Turner 2006). Animal farming and quality of the crops in the foraging home range affect the size and demographic trend of the colonies and have some weak effects on nestling phenotype (Møller 1994; Ambrosini *et al.* 2002; Turner 2006; Ambrosini *et al.* 2012). In our study population, 70–80% of the females lay four or five eggs per clutch. One to three clutches are laid from April to July. Most yearlings lay one clutch per year whereas most two- or more year old adults have two or three clutches, though the number of clutches may considerably vary among years and areas.

GENERAL METHODS

In 1993–2011, we studied 14 colonies (= farms) East and South-East of Milano (Northern Italy) that were regularly visited to record clutch size and hatching date (= date of hatching of the first egg to hatch) by inspecting the nests directly or by means of a mirror mounted on a pole, ring the nestlings and measure body mass, tarsus length and response to a standard test of T cell-mediated immunity ('PHA test'; see Saino, Calza & Møller 1997; Tella *et al.* 2008) at 10–13 days after hatching (see Statistical Analyses for an account of how we dealt with the potentially confounding effect of age). Because in several years we focused our nestling ringing effort on first clutches, most (>85% depending on the analysis) of the recruits considered here originated from a first clutch. Restriction of the sample to these individuals did never qualitatively alter the results (i.e. the effects that were significant on the entire data set remained such) compared to the analyses on the entire sample. We aimed at analyzing the effect of phenotypic quality of individual nestlings *relative* to their siblings on longevity. We therefore computed *relative* phenotypic values of body mass, size and immune response for individual recruits as the difference between the *absolute* value measured for that recruit and the mean of its brood of origin. As experiments have been carried out in the study population, all the recruits and adults from manipulated groups were excluded.

Our data consist of longevity of individuals that were marked as nestlings and were recruited as 1-year old breeding adults. Because natal dispersal is high (Turner 2006; Balbontín *et al.* 2009), to increase sample size we used data from our long-term study while appropriately testing for cohort effects. Recruits could be either 'local' (i.e. breeding in their colony of origin) or 'emigrants' (breeding in a different colony).

In all study years, we did repeated capture sessions in each farm throughout spring and summer (late March–July) to record recruitment and survival. Because barn swallows spend the night inside the buildings where they breed, we could capture all the individuals by putting up nets at all doors and windows before dawn. Importantly, barn swallows in our and neighbouring populations (North-western Italy; Southern Switzerland; see Data S1) show extremely high breeding philopatry (see also Møller 1994; Saino *et al.* 1999, 2011). High breeding philopatry and capture efficiency imply that recapture probabilities were homogeneous and very close to 1. The case that in few instances death was imputed for birds that in fact skipped breeding cannot be ruled out, although this eventuality was likely to be rare, as nonbreeding individuals are regularly captured at the colonies. As adult breeders do not disperse, longevity of recruits could be estimated accurately based on the information on year of recruitment (1 year of age) and of year of disappearance (i.e. the first year when they were not captured, implying that they had died) (see Møller 1994; Saino *et al.* 1999, 2011; see also Data S1).

Barn swallows leave the colonies in June-August and survivors return in March–May, implying that exact date of death was unknown. In modelling longevity we thus assumed the year of recruitment as time 0 and imputed longevity of 0·5 years to individuals that did not survive to age 2, 1·5 to those that survived to age 2 but not to age 3, and so forth. Hence, there was no left censoring in our data, whereas right censoring occurred for only 5·7% of the observations.

Breeding pairs were assigned to their nest by direct observation of breeding behaviour of the individuals that could be recognized, thanks to unique combinations of colour rings and markings on breast and belly feathers that were applied at first capture in any given year (see e.g. Saino *et al.* 1999). We could thus obtain information on breeding performance through the entire life (i.e. until the last year when they were captured) on a subsample ($n = 54$ individuals; 45 males; 9 females) of the recruits that could be included in the present study. As a proxy for LRS we used total number of eggs because this datum was available for a larger number of nests and it was collected in a more standardized way, as number of fledglings could not always be recorded at the same breeding stage. However, in different samples from several years and the same area, clutch size has been found to be highly positively correlated ($r > 0{\cdot}85$) with number of fledglings, as expected based on very low mortality in barn swallow broods (e.g. Boncoraglio, Caprioli & Saino 2009; see also Møller 1994; Cramp 1998; see also above).

STATISTICAL ANALYSES

We used accelerated failure time (AFT) and frailty models to investigate variation in longevity in relation to hatching date, brood size and nestling phenotype (covariates), cohort (= year of birth; multilevel factor) and sex (binary factor) (see Data S1). In the models, we entered the phenotypic values of individual recruits relative to their brood's mean and also the mean phenotypic value of the brood as a covariate. Hence, an effect of relative phenotypic values implies that the phenotypic quality of an offspring relative to its siblings predicts longevity independently of average phenotypic quality of the brood. Conversely, a significant effect of the mean brood value while accounting for relative phenotypic quality would imply that offspring of higher absolute quality are more viable (see Van de Pol & Wright 2009 for a discussion of within- vs. between-broods effects). We assumed a Weibull distribution of longevity (D) because the models with a gamma distribution often failed to converge and alternative distributions of D provided a poorer fit. The σ parameter in the models (see Data S1) accounts for any change in error variance, so that when $\sigma > 1$ the instantaneous risk (hazard) of death decreases with time, when $0{\cdot}5 < \sigma < 1{\cdot}0$ the hazard of death increases at a decreasing rate, while $0 < \sigma < 0{\cdot}5$ indicates that the hazard of death increases at an increasing rate. Cox regression models provided a poorer fit of longevity data. Because breeding phenology of the population may vary among years depending on ecological conditions (e.g. weather), relative hatching date (i.e. the difference between hatching date of the focal swallow and the population mean in that year) rather than absolute hatching date may be seen as a better indicator of the conditions under which an individual was reared. We thus re-ran the model of longevity using relative hatching date. In models of longevity in relation to offspring phenotypic traits, inclusion of the effect of age at measurement did never alter the quality of the results (i.e. the statistically significant or, respectively, nonsignificant effects remained such).

Because spatial heterogeneity has been acknowledged with a major role in determining population dynamics and viability (e.g. Bearhop, Ward & Evans 2003) and breeding habitat apparently varies in quality among barn swallow colonies (see above), we also used a frailty modelling approach to test for spatial structuring in longevity (e.g. Fox *et al.* 2006). Briefly, frailty terms modelling accounts for sources of variation due to unmeasured covariates by including a 'random' term which statistically accounts for intragroup dependence in hazard (see Fox *et al.* 2006).

In all models we accounted for right censoring of some (5·7%) observations. The 157 recruits that represent the overall sample of birds included in the study were derived from 148 different broods, implying that data dependence due to kinship was negligible. All analyses were run using PROC LIFETEST, LIFEREG and NLMIXED in SAS 9.2 (Allison 2010).

Results

The dataset included 157 (131 males; 26 females) recruits. Longevity data for nine individuals were right-censored (see Methods). The proportion of male recruits largely and significantly exceeded the null expectation based on an even nestling sex ratio (see Saino, Martinelli & Romano 2008), and this was the case when all recruits (goodness-of-fit $\chi^2_1 = 70{\cdot}22$, $P < 0{\cdot}001$) and when only local recruits (goodness-of-fit $\chi^2_1 = 66{\cdot}73$, $P < 0{\cdot}001$) were considered.

There was no significant difference in longevity between males and females in an AFT model where the effect of sex was entered as the only predictor ($\chi^2_1 = 0{\cdot}34$, $P = 0{\cdot}559$). Comparison of the survivor functions by the actuarial method again provided no evidence for sex-related variation (Wilcoxon test; $\chi^2_1 = 0{\cdot}01$, $P = 0{\cdot}913$) (Fig. 1a). In addition, while local recruits (119 males; 22 females) had somewhat reduced longevity compared to emigrants (12 males; 4 females) (Fig. 1b), the difference between the two groups was statistically nonsignificant ($\chi^2_1 = 2{\cdot}72$, $P = 0{\cdot}099$), and the same held true ($\chi^2_1 = 2{\cdot}75$, $P = 0{\cdot}097$) when we controlled for the effect of sex.

Longevity significantly declined with hatching date, with no significant differences between sexes (Table 1; Fig. 2a). The parameter estimated for hatching date (Table 1) yields a reduction in longevity of 0·58 years for an increase in hatching date of 2 standard deviations (1 SD = 18 days). In turn, 0·58 years correspond to *c.* 50% of life expectancy estimated at age 1. The Weibull scale parameter (σ) comprised between 0·5 and 1 (Table 1) implies that the hazard of death was increasing with time but at a decreasing rate. When we ran the model in Table 1 with relative hatching date (see Statistical Analyses) as a predictor, the results were qualitatively similar (effect of relative hatching date: $\chi^2_1 = 19{\cdot}94$, $P < 0{\cdot}001$, coefficient = −0·017 (0·004); sex: $\chi^2_1 = 0{\cdot}85$, $P = 0{\cdot}36$).

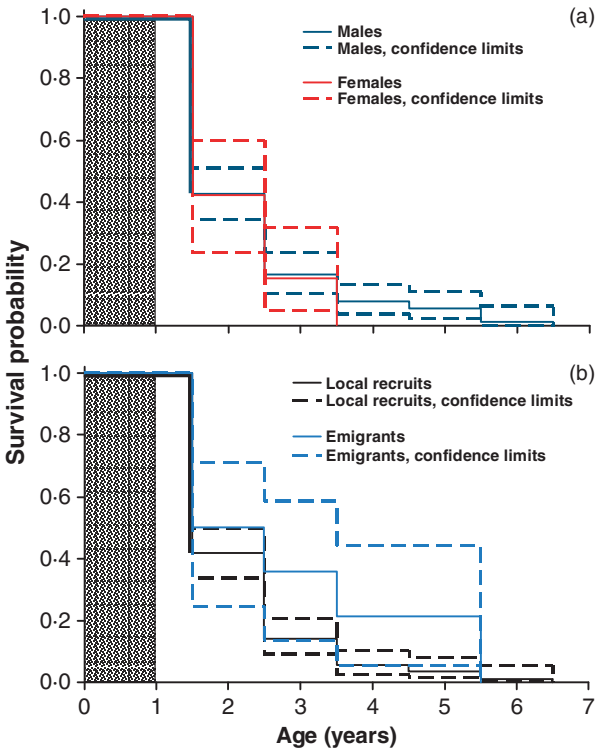

Fig. 1. Survivor functions and 95% confidence limits for a) male ($n = 131$) and female ($n = 26$) recruits and b) local ($n = 141$) or emigrant ($n = 16$) recruits. Age 1 year is the first spring after that of birth, when the individuals were recovered as recruits. Few individuals ($n = 9$; 5·7%) with right-censored observations are not shown to improve graphical clarity.

In AFT models (see Table 1), mean brood phenotypic values were never found to significantly predict longevity. In the same models, relative tarsus length did not significantly predict longevity. However, relative body mass had a differential effect on longevity of either sex, as females but not males benefited from being large relative to their siblings (Table 1). Moreover, nestlings that had large immune response relative to their siblings enjoyed significantly larger longevity, independently of sex (Fig. 2b). An inspection of Fig. 2b clearly shows that high immune response differentiated the individuals that survived up to age 2 years or more from those that did not. Hence, the results from the models including both relative and mean brood phenotypic values imply that recruits' relative but not absolute phenotypic values predicted longevity. Simplified models relating longevity to offspring-relative phenotypic values confirmed the results in Table 1 (details not shown for brevity). Similarly, models relating longevity to absolute phenotypic values always confirmed the lack of effect on longevity (details not shown for brevity). Brood size did not predict longevity either per se or in combination with the effect of sex ($P > 0{\cdot}88$ in both tests; other details not shown).

An AFT model simultaneously including the main terms of sex and all the covariates accounting for *relative* individual phenotypic values listed in Table 1 and brood size confirmed the significant negative effect of hatching date ($\chi^2_1 = 14{\cdot}00$, $P < 0{\cdot}001$) as well as the positive effect of immune response ($\chi^2_1 = 4{\cdot}94$, $P = 0{\cdot}026$) on longevity in a subset of 83 individuals for which information on all traits was available.

When we scrutinized the data for any cohort effect, in an AFT model there was no effect on longevity ($\chi^2_{13} = 12{\cdot}12$, $P = 0{\cdot}518$). Moreover, in different models longevity of different cohorts was not differentially affected by hatching date (interaction: $\chi^2_{11} = 16{\cdot}03$, $P = 0{\cdot}140$), relative tarsus length ($\chi^2_{10} = 13{\cdot}61$, $P = 0{\cdot}192$), body mass ($\chi^2_{10} = 10{\cdot}29$, $P = 0{\cdot}415$) or immune response ($\chi^2_9 = 7{\cdot}50$, $P = 0{\cdot}585$). Hence, the effects of hatching date and immunity on longevity were consistent among cohorts.

Finally, we investigated structured heterogeneity of longevity data in frailty models including sex and hatching date and the frailty term of colony of origin. Inclusion of farm of origin caused a reduction in deviance of 53·1 (residual deviance = 306·3) compared to the model including sex and hatching date only (see also Table 1). Hence, there was large spatial variance in longevity at the among-colonies level.

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Table 1. Weibull accelerated failure time (AFT) models of longevity in relation to hatching date or relative individual phenotype, expressed as deviation from the brood mean. In the models with offspring phenotype, within-brood mean was also included as a covariate. Log-likelihood values are given for the model with main effects only (first value for each variable), for the model also including the interaction between phenotypic value and sex (second value), or for the null model. Differences in deviance between the three models are also given. χ^2 and *P*-values of main effects refer to the model excluding the interaction, except for body mass where the interaction was significant

AFT model	−2 log likelihood	d.f.	Deviance	Residual d.f.	Wald χ^2	*P*
Hatching date[a]	359·42	1	21·62	154	24·85	< 0·001
Sex		1			0·46	0·497
Hatching date × sex	359·17	1	0·25	153	0·25	0·614
Null	381·04					
Relative tarsus length	275·01	1	1·65	110	0·04	0·833
Sex		1			0·35	0·557
Mean brood tarsus length		1			1·57	0·210
Relative tarsus length × sex	273·12	1	1·89	109	1·80	0·180
Null	276·66					
Relative body mass	274·72	1	2·27	110	4·00	0·046
Sex		1			1·83	0·176
Mean brood body mass		1			2·87	0·090
Relative body mass × sex[b]	268·74	1	5·98	110	6·02	0·014
Null	276·99					
Relative immune response[c]	195·55	1	10·73	83	7·24	0·007
Sex		1			0·01	0·909
Mean brood immune response		1			2·89	0·089
Relative immune response × sex	194·14	1	1·41	82	1·23	0·268
Null	206·28	1				

Sample size of recruits was as follows: hatching date: 157; tarsus length: 114; body mass: 115; immune response: 87. Range of σ values was 0·693–0·754.

[a]Coefficient from the model including main effects only = −0·0164 (0·0033 SE).

[b]Coefficient for males = −0·036 (0·087 SE), χ^2 = 0·17, *P* = 0·681; females = 0·470 (0·193 SE), χ^2 = 4·96, *P* = 0·015.

[c]Coefficient from the model including main effects only = 0·641 (0·238 SE).

In a subsample of 45 males, LRS could be measured. As expected from the negative relationship between hatching date and longevity, and the positive effect of age on number of clutches laid per season (see Methods), there was a significant negative relationship between LRS and hatching date in a linear regression analysis ($F_{1,43}$ = 9·76, *P* = 0·003, coefficient = −0·20 (0·07); Fig. 3). The sample of nine females was too small to be amenable to statistical analysis.

Discussion

Longevity shows considerable variation within populations of organisms, but there is little knowledge of the mechanism that may operate early in life in producing such variation (Cam, Monnat & Hines 2003; Van de Pol *et al.* 2006; Ricklefs & Cadena 2008; Wilkin & Sheldon 2009; Bouwhuis *et al.* 2010a,b). Long-term consequences of perinatal conditions on longevity are indeed to be expected, however, given the importance of environmental and early maternal effects in shaping offspring life history (see Introduction).

We thus investigated longevity of barn swallows and found that it declined with hatching date, independently of any cohort effect. Moreover, we could show for the first time in any bird species that nestlings that mounted an intense T cell-mediated immune response relative to their siblings and female nestlings that were high in the size/age brood hierarchy had larger longevity. Our study focused on longevity of individuals that had reached sexual maturity, thus by-passing the effect of intense selection during the first year of life (e.g. Møller 1994; Turner 2006; Grüebler & Naef-Daenzer 2008). Importantly, hatching date negatively predicted LRS, although this relationship could be tested only in males due to small sample size of females, which ultimately depends on heavy sex bias in natal dispersal distance. Such negative relationship between LRS and hatching date could be expected based on the observed negative relationship between longevity and hatching date. In fact, most barn swallows have a single clutch in their first breeding season, whereas most individuals have two (seldom three) clutches per breeding season when two- or more years old. Thus, broodedness increases with age in this species where more than 80% of the individuals that reach sexual maturity have only two breeding seasons (see Fig. 1). Moreover, variance in clutch size is relatively small (see Methods). Finally, more than 90% of the individuals breed every year (our unpublished data; see also Møller 1994). Hence, a major mechanism accounting for variation in Darwinian fitness according to longevity is larger broodedness of older individuals. As reduction in longevity and LRS

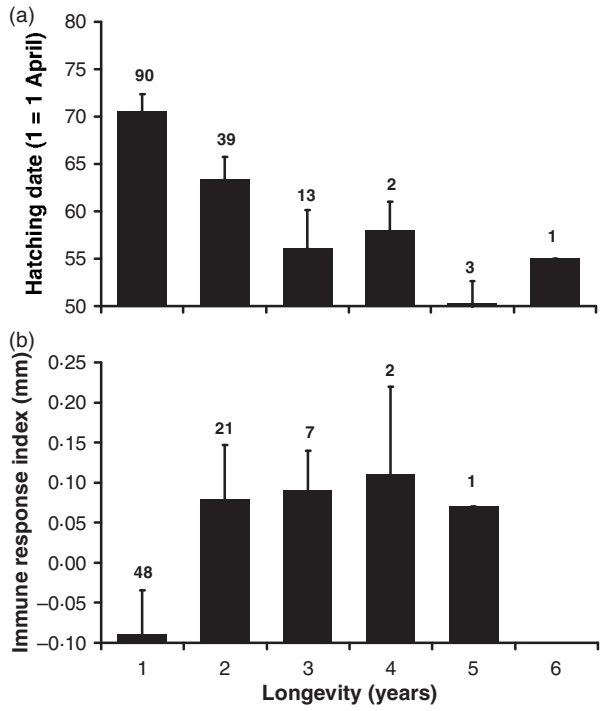

Fig. 2. Mean (+SE) hatching date (a) or T cell-mediated immune response (b) of recruits with different longevity relative to their siblings. Immune response data are expressed as deviation from the mean of the brood to which the individual recruits belonged. Hence, negative values indicate that a recruit's immune response was smaller, whereas positive values indicate that it was larger than that of its siblings. Longevity = 1 indicates the individuals that died between their first (= age 1 year) and second (= age 2 years) breeding seasons. Longevity 2 indicates individuals that died between age 2 and 3, and so forth. Numbers above bars are sample sizes. The nine censored individuals are excluded from the calculations.

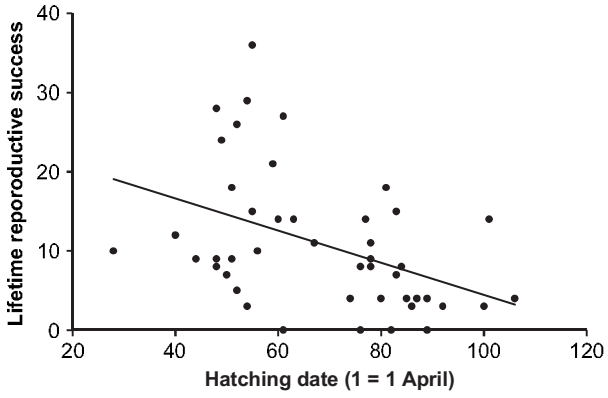

Fig. 3. Lifetime reproductive success (= total lifetime number of eggs in their nests) in relation to hatching date of 45 male recruits. Line is fitted by simple regression analysis.

with hatching date was marked, present results imply that selection for early breeding is intense.

Birds at temperate latitudes experience seasonal worsening of environmental conditions during the breeding season (Lack 1950; Turner 2006; see Introduction), which can cause a progressive deterioration of offspring quality and viability (Newton 1989; Møller 1994; Grüebler & Naef-Daenzer 2008). Constraints on early breeding set by migration schedule, particularly on European trans-Saharan migrants like the barn swallow (McNamara & Houston 2008; Newton 2008; Møller, Fiedler & Berthold 2010), may exacerbate these negative seasonal effects, with long-term repercussions on viability after sexual maturation. In addition to extrinsic factors, the phenotypic quality of parents may also decline late in the breeding season. In fact, conditions experienced during wintering and migration, which are known to predict interannual variation in spring arrival date and breeding performance of barn swallows (Saino *et al.* 2004), may carry-over to affect physiological state of individuals that adopt different migration strategies, generating variance in parental quality. Because individuals in prime condition arrive and thus breed early (Møller 1994), a decline in longevity with hatching date may reflect a negative covariation between parental quality and arrival/breeding date. Overall, the negative relationship between longevity and hatching date is compatible both with an effect of parental quality and of seasonal effects, and these two alternative sources of variation may be difficult to tease apart, because quality of adults and their breeding date may be strongly correlated, and the flow of causation between these variables may be ancipital. Indeed, low parental quality may entail a late breeding because, for example, it causes a late arrival. Conversely, adults that for any reason unrelated to their inherent quality are forced to breed late under relatively adverse conditions may, because of this, perform poorly at parental duties. Two lines of evidence, however, suggest that parental age/quality was unlikely to be the major (or sole) determinant of variation in offspring longevity. First, two- or more years old adults breed earlier but offspring quality declines already after the first or second year of life (Møller 1994; Saino *et al.* 2002b). Second, we observed that relative rather than absolute phenotypic values of the offspring predicted longevity. Hence, within-brood rather than among-broods (and thus among-parents) variation in offspring quality affects longevity.

The specific mechanisms that cause a decline in longevity with hatching date are a matter of speculation. Egg quality is a candidate as a vector of long-term maternal effects on longevity (Beamonte-Barrientos *et al.* 2010). Biochemical egg quality in birds can vary seasonally (López-Rull, Salaberria & Gil 2010), and experiments have disclosed short-term effects of egg quality on offspring phenotype but also 'developmentally entrenched' (see Badyaev 2008) long-term effects of egg antioxidants, immune factors and maternal hormones which carry-over into adulthood (Groothuis *et al.* 2005; Badyaev *et al.* 2006; Rubolini *et al.* 2007; Bonisoli-Alquati *et al.* 2011). In mammals, prenatal and early postnatal exposure to low-quality diets is causally linked to ageing and longevity (Sayer *et al.* 2001). The quality and amount of food received during the pre- and postfledging stages, when development of some physiological functions (e.g. immunity) is still under way, is an obvious additional candidate actor of maternal effects on longevity (De Kogel 1997). Other mechanisms

may involve seasonal variation in parasitism or carry-over effects on autumn migration, which may in turn impose viability costs later in life (Mërila & Svensson 1997; Fitze, Clobert & Richner 2004). In addition, antioxidant protection strongly predicts annual survival of barn swallows (Saino *et al*. 2011; see Bize *et al*. 2008) and may decline seasonally (Costantini, Carello & Fanfani 2010). Poor egg or dietary antioxidants supply can have long-lasting detrimental effects on antioxidant absorption and protection from oxidative damage later in life (Monaghan, Metcalfe & Torres 2009). Hence, impaired antioxidant defence is a potential mechanism linking hatching date to longevity.

Nestlings with superior immunity and body mass (females only) relative to their siblings enjoyed larger longevity, suggesting that prevailing in brood competition for food has long-term, besides obvious short-term, effects on viability (Mock & Parker 1997). In barn swallow broods, even small hatching asynchrony determines a size/age hierarchy that is consistently retained throughout the nestling period. Present results thus highlight the long-term positive fitness consequences of hatching early relative to siblings and acquiring a dominant position in the size hierarchy (Ricklefs 1993) particularly for females, which are competitively inferior to males in barn swallow broods (Boncoraglio, Martinelli & Saino 2008). Because functioning of the immune system is strongly dependent on nutritional conditions (Klasing 2007), a positive effect of immune response on longevity may also reflect the general consequences of nutrition on viability. A positive effect of T cell-mediated immune response and body mass on longevity after sexual maturation is consistent with the observation that these traits predict local recruitment in several birds (e.g. Møller & Saino 2004; Cleasby *et al*. 2010; López-Rull *et al*. 2011). A differential effect of rearing conditions on longevity and breeding success has been observed also in great tits (*Parus major*), where males but not females benefitted from being raised under benign conditions (Wilkin & Sheldon 2009). In the present study, females, not males, benefitted from ranking high in the brood size/age hierarchy, possibly because position in the hierarchy is more critical to the competitively inferior females.

Sex ratio of barn swallow nestlings is *c*. 1:1, though with some interannual variation (Saino, Martinelli & Romano 2008). Similarity in longevity of adult males and females observed here therefore suggests that male-biased tertiary sex ratio is caused by larger mortality of females during the first year of life. An alternative, though unlikely, explanation is that the postnatal dispersal/immigration balance differs between sexes in the population we studied.

Dispersal did not predict longevity, consistently with observations on Danish swallows, but in contrast with the reduced viability of male emigrants in Spain (Balbontín *et al*. 2009; see Bouwhuis *et al*. 2010b). If anything, emigrants had nonsignificantly larger longevity, implying that viability costs of dispersal vary geographically. However, we caution that the analyses of dispersal concerned a small number of emigrants, reducing the power of the statistical test.

Much of the variance in longevity remained unexplained by offspring traits. This is not a surprise because barn swallows are long-distance migrants on which sources of mortality unrelated to perinatal conditions likely operate. Inclusion of the frailty effect of colony of origin, however, led to a considerable reduction in the model's deviance, again stressing the importance of rearing conditions in determining longevity. Spatially structured heterogeneity in longevity has consequences for analyses of population trends and viability (see Bearhop, Ward & Evans 2003). The barn swallow is a common farmland species whose populations have markedly declined throughout Europe (BirdLife International 2004) as well as in our study area ($-8{\cdot}4\%$ year^{-1}; Ambrosini *et al*. 2012). Present results demonstrate that spatial heterogeneity in a major demographic trait is large and may need to be incorporated in analyses of demographic trends.

In conclusion, this is the first study of birds we are aware of showing that longevity after sexual maturity depends on hatching date and on phenotypic quality of nestlings *relative* to brood mates, although other studies have provided evidence for effects of rearing conditions on LRS and longevity. This supports the idea that early environmental and maternal effects mediated by decisions on the time of breeding and resource allocation can have long-term consequences on major fitness trait such as longevity after sexual maturation and LRS. Although their mechanisms are unknown, these effects afford a link translating parental reproductive decisions into variation in population demographic parameters and viability. Finally, we showed that spatial structuring in longevity exists at the inter-colony level, which may need to be incorporated into demographic models of this declining bird.

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Supporting Information

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Data S1. General methods.

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SUPPORTING INFORMATION

General methods

Because tarsus length, body mass and the immune response index were not routinely recorded in all nests in the starting years of the study, the information on these traits is missing for part of the sample (see Results for sample sizes).

Extremely high breeding philopatry (implying that an individual that has bred in a farm in a given year does not move to other farms to breed in the following years) has been documented in our long-term study of the same population (see Saino et al., 1999; see also Saino et al., 2011), with only two cases reported over several thousands of individual x year records. Similar evidence has been collected in a recent study in another area in Northwestern Italy, with only 1 bird out of more than 400 adults moving to a different, very close farm (our unpubl. data), as well as in Southern Switzerland (C. Scandolara, pers. comm.).

Statistical analyses

We relied on accelerated failure time (AFT; Kalbfleisch and Prentice, 1980) and frailty models (Duchateau and Janssen, 2008) to investigate variation in longevity in relation to hatching date, brood size and phenotype (covariates), cohort (= year of birth; multilevel factor) and sex (binary factor). In their most general form, AFT models account for any relationship between the survivor functions of any two given individuals. Specifically, we modeled death time D_i for any *i*-th recruit in our sample as

$$Log(D_i) = \beta_0 + \beta_1 x_{i1} + \ldots \beta_k x_{ik} + \sigma\varepsilon_i$$

where $\beta_0 \ldots \beta_k$ and σ are parameters to be estimated, ε is a random error term, and $x_1 \ldots x_k$ are k covariates measured on any *i*-th recruit.

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Chapter 7

Early exposure to a bacterial endotoxin may cause breeding failure in a migratory bird

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Early exposure to a bacterial endotoxin may cause breeding failure in a migratory bird

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ABSTRACT

Early life conditions may have important long-term consequences on viability and reproduction. Considering their pervasiveness in natural environments, parasites play a crucial role in determining life-histories of hosts, by negatively affecting their performance and imposing fitness costs. Here, we tested whether exposure to bacterial endotoxin (lipopolysaccharide, LPS) during the nestling period influenced breeding performance at sexual maturation of a small passerine bird, the barn swallow (*Hirundo rustica*). Three out of 56 LPS male nestlings were recruited in their breeding site, compared to 6 out of 51 controls. None of the LPS recruits was observed to breed or to pair with a female, while all control recruits bred successfully, with a mean seasonal breeding success of 6.4 fledged offspring. In addition, LPS recruits were captured later than controls. These findings suggest that an exposure to a bacterial endotoxin during the critical phase of development may negatively affect reproductive success in this short-lived migratory birds, possibly via delaying timing of moult and arrival from wintering grounds.

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INTRODUCTION

Conditions experienced during early life are not only fundamental in moulding individual development, but may also affect phenotype in the long-term (reviewed in Metcalfe and Monaghan, 2001), with consequences on viability and reproduction (Lindström, 1999; Ricklefs et al., 2006). In avian species, several, diverse factors, including parental quality (Naguib et al., 2006), natal habitat (van de Pol et al., 2006 Saino et al., 2012), time and duration of rearing period (Cam et al., 2003), as well as nutritional conditions (Birkhead et al., 1999; Blount et al., 2006; Naguib and Nemitz, 2007) and levels of oxidative damage during early development (Noguera et al., 2012), have been related with longevity, sexual attractiveness and breeding performance in adulthood. Moreover, early attack by parasites may considerably reduce host fitness (Loyle and Zuk, 1991; Møller, 1997). For example, beside direct detrimental costs, parasites may increase energy expenditure by stimulating immune system functioning, resulting in a trade-off with other body functions (Bonneaud et al., 2003; Faivre et al., 2003; Soler et al., 2003). Despite hosts having evolved several defence mechanisms to cope with virulent parasite attacks, parasites can markedly influence development of young animals or affect adults in diverse ways, by e.g. reducing annual survival and reproduction or modifying the expression of secondary sexual characters (Loyle and Zuk, 1991; Møller, 1997).

In natural environments, bacteria are common parasites to which birds are exposed at all stages of their life cycle and may cause detrimental effects on condition, viability and reproduction (reviewed in Benskin et al.,

2009). Here, we tested the long-term consequences of exposure to a bacterial endotoxin (lipopolysaccharide from *Escherichia coli* cell walls, LPS) of barn swallow (*Hirundo rustica*) nestlings on their reproductive success when they were recruited in their original colony as breeding, 1-year old adults. We previously demonstrated that LPS negatively affected body growth, feather quality and behaviour of nestlings, but had no consequences for pre-fledging survival (Romano et al., 2011).

The barn swallow is a small, insectivorous and socially monogamous migratory passerine, which usually breeds in colonies located in rural buildings, such as stables and cowsheds. Females lay from one to three clutches of 3-7 eggs per breeding season and both parents perform parental care. Nestlings fledge approximately 18-20 day after hatching. Natal dispersal is high: in our study population, recruitment rate in the natal colony is <5% and strongly male-biased (Saino et al., 2012). We therefore focused on fitness consequences for males only.

MATERIALS AND METHODS

This study was performed in 2010 and 2011 in two colonies (= farms) located near Milan (Italy). In April-July 2010, at day 7 (day 0 = hatching of the first egg in a nest) we individually ringed all nestlings from 47 broods and collected a blood sample for molecular sex determination (see Romano et al., 2011 for details). The present study was carried out on the same set of nestlings that were analyzed in Romano et al. (2011). This procedure allowed us to know the nestlings' sex before day 12 after hatching, when 20 µl of phosphate buffered saline (PBS) containing 10 µg of LPS (055:B5–L2880 Sigma-Aldrich) were intraperitoneally injected to half of the nestlings of either sex within each brood (56 males and 46 females; hereafter 'LPS' individuals) to simulate a bacterial infection. The remaining 100 nestlings (51 males and 49 females) were injected with the same amount of PBS (hereafter 'control' individuals). Nestlings were assigned to either group randomly. In case of an odd number of male or female nestlings, the odd nestling was assigned randomly to either treatment (see Romano et al., 2011). For each nestling we collected morphological data (body mass, tarsus length, third outermost wing feather length, outermost tail feather length and number of fault bars on feathers) before (at day 12) and after (at days 14 and 15) the LPS injection (see Romano et al., 2011).

In April-August 2011, we visited the colonies on a weekly basis to record breeding events. At each colony, we performed three capture sessions (ca. one session every 4-5 weeks) of adults during the breeding season (May 4 – July 12) by placing mist nets before dawn at windows and doors of all rooms accessible to barn swallows. As adults generally spend the night inside the buildings, this procedure assures high efficiency of capture of breeding and non-breeding individuals (less than 5% of individuals can escape repeated capture events; see Saino et al., 2011 for details). At capture, standard measurements of morphology and infestation by ecto-parasites were taken: we collected data of body mass, tarsus length, wing length, outermost tail feather length, and number of holes on feathers caused by chewing lice (see

e.g. Vas et al., 2008). Before being released, adults were individually marked with a unique combination of colour patches on belly and breast feathers and colour rings. This procedure allowed us to assign each individual to its breeding pair and nest by direct observation. We then recorded the number of nestlings fledged at each nesting attempt.

RESULTS AND DISCUSSION

Overall, 6 out of 51 control and 3 out of 56 LPS males were recruited in their natal colony. All recruits were from different nests. No females were recruited. At recruitment, morphology and ecto-parasite load of LPS and control males did not differ significantly (**Table 1**). Phenotypic traits of recruited birds were also similar when they were measured as nestlings, both before (day 12; for all traits: t-test for unequal variances: $P > 0.26$) and after (day 14 and 15; for all traits and ages: t-test for unequal variances: $P > 0.09$) the LPS injection.

Table 1. Mean (SE) values of morphological traits and parasite load of LPS and control individuals at recruitment (ca. 1 year of age). No significant differences emerged between groups after *t*-tests assuming unequal variances (all *P*-values > 0.59).

	Control	LPS
Body mass (g)	16.63 (0.14)	17.03 (1.03)
Tarsus length (mm)	11.14 (1.04)	10.95 (2.86)
Wing length (mm)	121.83 (1.30)	121.67 (1.76)
Tail feather length[a] (mm)	99.50 (2.29)	98.67 (1.20)
Chewing lice holes[b]	14.00 (3.85)	14.30 (9.49)

a: Outermost tail feather length (mm)
b: Number of chewing lice holes on feathers

None of the 3 LPS males was found to breed or even to pair with a female, whereas all 6 control recruited yearlings did, resulting in a statistically significant difference in the chances of breeding between the two groups (Fisher's exact test: $P = 0.012$). Over the whole breeding season, the mean fledging success of the control males was 6.4 offspring (range 3-10), statistically larger than that of LPS recruits (Mann-Whitney U test: $Z = 2.306$; $P = 0.021$). While all control recruits were captured during the first capture session, this was the case for only a single LPS recruit, resulting in a statistically significant difference in ranks of capture date between groups (Mann-Whitney U test: $Z = 2.121$; $P = 0.034$).

These results suggest that an exposure to LPS endotoxin during development may negatively impact on individual fitness of barn swallow males. As life expectancy of yearling barn swallows is about 2 years, due to an annual mortality rate of about 60-70% (Turner 2006), a failure of the first reproductive attempt may thus seriously compromise lifetime reproductive success (see also Saino et al., 2012). Despite sample size was small because of the very low recruitment in the natal colony, we emphasize that, to our knowledge, this is the first study reporting that an exposure to bacteria in early life has long-term negative effects on reproductive success in free-living birds.

Two proximate non-exclusive mechanisms may have caused such breeding failure by the LPS recruits. First, it could be mediated by differences in arrival date of control and LPS males. Arrival date to the breeding colony is in fact the most important determinant of mating success in the barn swallow (Møller, 1994). In the study population, late arriving males (normally yearlings) may have less chances to mate than early arriving ones, since the tertiary sex ratio is usually, though not strongly, male-biased (Saino et al., 2008). This was the case

for the colonies where we conducted the present study in 2011, where we captured through the breeding season 51 females and 67 males. Since LPS is known to severely impair moult speed in passerines (Moreno-Rueda, 2010), delayed completion of moult in the winter quarters in Africa may have resulted in later arrival to the breeding grounds. In addition, LPS injection, by impairing juvenile plumage quality (see Romano et al., 2011), may have resulted in reduced flight and thus migratory performance (see also Criscuolo et al., 2011), ultimately causing a delayed timing of migration. The delayed capture of unpaired, LPS males is consistent with a delay in time schedules of LPS birds during the non-breeding season. However, we cannot exclude the possibility that such delayed capture was caused by a different behaviour of unpaired males, resulting in lower capture probability, though this seems unlikely as barn swallow males, including unpaired ones, establish and defend a small breeding territory inside rural buildings soon after arrival from wintering grounds (see Møller, 1994).

Secondly, breeding failure may have been mediated by a reduced male attractiveness, though the most important secondary sexual character subjected to directional female preference, the outermost tail feathers length (Møller, 1994), did not differ between control and LPS recruits (**Table 1**). However, other sexually-selected traits that convey reliably information about male quality not measured here, such as male song repertoire (Galeotti et al., 1997; Saino et al., 1997; Møller et al., 1998), may have been negatively affected by poor early life conditions, as documented in other bird species (Nowicki et al., 2002; Buchanan et al., 2003; Spencer et al., 2003). In addition, it has been previously demonstrated that LPS affects male sexual behaviour in the short-term, decreasing territorial aggression and vocal displays (Owen-Ashley et al., 2006), and negatively influences breeding performance of nestling birds (Bonneaud et al., 2003; Palacios et al., 2011).

Many, different ultimate mechanisms may have been involved in mating failure by LPS recruits. In birds, host response to LPS is known to alter several physiological, hormonal and behavioural traits, i.e. inducing stress hormones production, increasing oxidative tissue damage and reducing fat scores (see Owen-Ashley et al., 2006; Bertrand et al., 2006; Lee et al., 2005; Bonneaud et al., 2003). LPS injection may have thus negatively impacted on young birds by causing trade-offs between immune system activation and investment in other body functions at subsequent life stages (e.g. Moreno-Rueda, 2010; Bonneaud et al., 2003).

Previous studies documenting adverse effects of exposure to novel antigens on bird species showed a reduction in survival of both adults (Hanssen et al., 2004) and post-fledged juveniles (Eraud et al., 2009), whereas our findings show that negative fitness consequences may be mediated by a reduction of mating success. Thus, host response to bacterial infection during early life may have long-term detrimental effects not only on survival prospects, but also on breeding opportunities, with potentially severe negative consequences on lifetime reproductive success.

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DISCUSSION AND CONCLUSIONS

Synopsis and concluding remarks

Using both experimental and correlative approaches, the present thesis has investigated the factors, including context-dependent sibling interactions, differential parental investment according to sex and environment, and decisions by parents over the time and site of breeding, that can contribute to determine the variation of offspring quality and viability before fledging, and also later in life.

First, the dissertation provides novel findings about the function and the evolution of begging behaviour, performed both in the presence and in the absence of parents, as a reliable carrier of information about offspring state, and the ultimate role of begging displays in the resolution of sib-sib and parent-offspring conflicts. It further shows that begging strategies and the outcome of interactions among nestlings can be considerably affected by diverse factors, including, sex, immune stimulation, and own compared to siblings' hunger level.

Moreover, the present work also provides new pieces of evidence for the importance of perinatal environment and of parental decisions over timing and site of breeding in affecting nestling growth and reproductive value. Environmental conditions during the rearing period can potentially determine sex-related developmental asymmetries and pre-fledging mortality rates as well as long-term effects on survival prospects and lifetime reproduction, and influence the parental sex allocation strategies.

Below I present a brief summary of the main findings described in each chapter of the dissertation.

Begging, sibling competition and kin selection

The aim of this part (**Chapters 1-3**) was to disclose factors, both in the short- and in the long-term, involved in affecting the expression of multiple begging displays, in moulding the competitive behaviour and the begging strategies by interacting nestlings, and in influencing the allocation of food by parents in the barn swallow. In addition, in the same model species I have analyzed the occurrence and experimentally tested the potential function of the so-called parent-absent begging in mediating sibling competition for food.

In **Chapter 1**, I have provided evidence for a fine-tuned modulation of begging behaviour by nestlings in relation to sibling's need of food: less hungry nestlings considerably reduced their competitive effort when facing a sibling in poor condition. Such a decreased competitiveness favoured the access to food by needy kin and allowed them to recover from a food deficiency. The modulation of selfishness thus assured indirect fitness benefits by enhancing survival prospects of siblings, while concomitantly reducing costs of vigorous competition against hungry nest mates.

In **Chapter 2**, I have shown that the frequency of parent-absent begging (PAB) increased at increasing hunger level (i.e. after a nestling have been experimentally deprived of food), and that nestlings modulated their begging behaviour according to own and sibling's solicitation in the absence of parents. In particular, nestlings that had performed PAB increased their competitive effort during the subsequent feeding visit by parents, while siblings reduced their own begging level, but only if not deprived of food. The combined

variation in begging by the interacting siblings resulted in a larger chances of obtaining food by the nestling which has performed PAB, but not when it was competing with a food-deprived nest mate, which in turn tried to monopolize the food delivered by parents to the nest.

Taken together, the results of **Chapter 1** and **Chapter 2** therefore show that offspring are able to modulate their begging strategies in order to achieve an optimal balance between the costs of scrambling for food, the benefits of being fed according to their need and the indirect costs of subtracting food to relatives. In addition, the present findings emphasize the importance of kin selection in moulding the complex interactions among siblings for the access to limiting parental resources and show that offspring may play a crucial role in the control of allocation of parental investment.

In **Chapter 3**, I have shown that an immune challenge during early growth, simulating a bacterial infection, caused a decrease in body mass and plumage quality, measured as the occurrence of feather abnormalities (e.g. fault bars), as well as in the red coloration of the gape in nestlings of both sexes. Postural solicitation of parental care considerably increased when nestlings had been experimentally deprived of food and also when they had been subjected to an immune challenge, implying that begging behaviour can convey reliable information on both offspring hunger level and condition, possibly being exploited by parents to optimally modulate allocation of care. I have also demonstrated a sex-dependent variation in the effects of the immune challenge: females suffered a major decrease in quality after the experimental manipulation (e.g. reduction of feathers growth and flange colour), as also shown by differences in the expression of postural begging behaviour between the sexes. However, variation in general condition did not affect parental food allocation both before and after a period of food deprivation, likely because barn swallows usually adopt a 'brood survival' strategy whereby parents tend to promote successful fledging of all offspring rather than discriminating among their progeny.

Sex-related parental investment and ecological conditions

The aim of the second part (**Chapters 4-5**) of the thesis was to explore variation of parental investment towards male and female offspring according to the spatial and temporal variation of ecological conditions in two passerine birds, namely the barn swallow, a species with sexual monomorphic nestlings, and the common starling, where a sexual dimorphism occurs at the nestling stage.

In **Chapter 4**, I have shown that the proportion of male offspring that successfully fledged (i.e. secondary sex ratio) significantly increased in barn swallow colonies surrounded by large extent of hayfields, the main foraging habitat for this insectivorous bird, and, only in second broods, in colonies showing recent negative demographic trends. Irrespective of the mechanism at the basis on such observed skewed progeny sex ratio, these findings suggested patterns of adaptive sex allocation strategy by parents according to the sex-specific features of natal dispersal of the barn swallow. Indeed, barn swallow males have a higher natal philopatry than females and are

thus more likely to benefit from favourable local environment when, as 1-year old adults, they will be recruited to breed in the area surrounding their natal site.

In **Chapter 5**, I have documented a seasonal decline in offspring quality in the common starling. Antioxidant defense and body growth rate were lower in the second compared to the first broods, indicating that late breeding attempts can result in offspring of lower reproductive value, and therefore that the timing of reproduction is a crucial determinant of fitness. Such a decrease in condition was particularly intense for females, and was accompanied by female-biased mortality in the late-hatched broods, where a marked difference in size compared to male siblings occurred. Sexual dimorphism in body mass at halfway through the rearing period, typical of the species, markedly differed between broods, with second-brood males being 22.7% and first-brood males 4.7% heavier than their female siblings, respectively. This observed pattern was likely caused by a skew of parental investment towards larger, higher-quality males (by e.g. a variation in maternal effects before hatching or a biased food provisioning during rearing), or determined by asymmetric sibling competition whereby stronger male competitors can monopolize resources.

Long-term effects of rearing conditions on reproductive success

In the last part of the present thesis (**Chapters 6-7**), I have investigated the effects of environmental conditions, determined by parental decisions over breeding and presence of parasites, experienced during the nestling period on longevity and lifetime reproductive success at the adult stage (age $\geq$ 1 year) in the barn swallow.

In **Chapter 6**, in a long-term longitudinal study, spanning from 1993 and 2011, I have demonstrated that individuals of both sexes that hatched early in each breeding season can benefit from a longer lifespan and a larger number of offspring successfully reared during the entire life. Present results again indicate an increase of reproductive success for early-breeders, which are therefore favoured by natural selection. In addition, high individual quality at the nestling stage, measured as T-cell mediated immune response (both sexes) and body size compared to siblings (only for females), predicted lifespan. This finding therefore suggests that individual rank in the hierarchy within-brood can have not only important effects in affecting nestling's growth and pre-fledging survival prospects, but can also have long-lasting fitness consequences until adulthood. Acquiring a dominant position in the size hierarchy within-brood could be particularly relevant for females, that are competitive inferior compared to their male siblings.

Finally, in **Chapter 7**, I have found, in small sample of males, that a bacterial immune challenge during the early post-natal stage causes the failure of the first breeding attempt. All males subjected to an injection of a bacterial endotoxin during the nestling stage that were recruited in their natal site as sexually mature adults did not mate. Conversely, all control individuals (i.e. males subjected to an injection of a physiological solution during the nestling stage) recruited in the same natal site bred successfully. In addition, immune challenged individuals were captured at their

natal site during the breeding season later than control males. An early exposure to virulent bacteria can thus markedly reduce the reproductive success in this short-lived species, possibly by means of a delay in the timing of migration resulting in a late arrival in the breeding sites.

Appendix

List of publications in ISI-ranked journals by Andrea Romano

The list includes all the publications that I have co-authored during the years of my PhD. The most recent Impact Factor (IF) of each journal is reported.

11. Sicurella BC, Caprioli M, **Romano A**, Romano M, Rubolini D, Saino N, Ambrosini R (in press) Hayfields enhance colony size of the barn swallow *Hirundo rustica* in Northern Italy. *Bird Conservational International* (IF: 1.250).

10. Rubolini D, Colombo G, Ambrosini R, Caprioli M, Clerici M, Colombo R, Dalle Donne I, Milzani A, **Romano A**, Romano M, Saino N (2012) Sex-related effects of reproduction on biomarkers of oxidative damage in free-living barn swallows (*Hirundo rustica*). *PLoS One* **7**: e48955 (IF: 4.092).

9. Saino N, Romano M, Caprioli M, Ambrosini A, Rubolini D, Scandolara C, **Romano A** (2012) A ptilochronological study of carry-over effects of conditions during wintering on breeding performance in the barn swallow. *Journal of Avian Biology* **43**: 513–524 (IF: 2.28).

8. **Romano A**, Caprioli M, Boncoraglio G, Saino N, Rubolini D (2012) With a little help from my kin: barn swallow nestlings modulate solicitation of parental care according to nestmates' need. *Journal of Evolutionary Biology* **25**: 1703–1710 (IF: 3.276).

7. Saino N, Romano M, Ambrosini R, Rubolini D, Boncoraglio G, Caprioli M, **Romano A** (2012) Longevity and lifetime reproductive success of barn swallow offspring are predicted by their hatching date and phenotypic quality. *Journal of Animal Ecology* **81**: 1004–1012 (IF: 4.937).

6. Ambrosini R, Rubolini D, Trovò P, Liberini G, Bandini M, **Romano A**, Sicurella BC, Scandolara C, Romano M, Saino N (2012) Maintenance of livestock farming may buffer population decline of the barn swallow *Hirundo rustica*. *Bird Conservational International* **22**: 411–428 (IF: 1.250).

5. Caprioli M, Ambrosini R, Boncoraglio G, Gatti E, **Romano A**, Romano M, Rubolini D, Gianfranceschi L, Saino N (2012) *Clock* gene variation is associated with breeding phenology and may be under directional selection in the migratory barn swallow. *PLoS One* **7**: e35140 (IF: 4.092).

4. **Romano A**, Ambrosini R, Caprioli M, Bonisoli-Alquati A, Saino N (2012) Secondary sex ratio covaries with demographic trends and ecological conditions in the barn swallow. *Evolutionary Ecology* **26**: 1041–1053 (IF: 2.453).

3. Serra L, Pirrello S, Caprioli M, Griggio M, Andreotti A, **Romano A**, Pilastro A, Saino N, Sacchi R, Galeotti P, Fasola M, Spina F, Rubolini D (2012) Seasonal decline of offspring quality in the European starling *Sturnus vulgaris*: an immune challenge experiment. *Behavioral Ecology and Sociobiology* **66**: 697–709 (IF: 3.179).

2. **Romano A**, Rubolini D, Caprioli M, Boncoraglio G, Ambrosini R, Saino N (2011) Sex-related effects of an immune challenge on growth and begging behavior of barn swallow nestlings. *PLoS One* **6**: e22805 (IF: 4.092).

1. Saino N, Caprioli M, Romano M, Boncoraglio G, Rubolini D, Ambrosini R, Bonisoli-Alquati A, **Romano A** (2011) Antioxidant defenses predict long-term survival in a passerine bird. *PLoS One* **6**: e19593 (IF: 4.092).

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